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HUMAN ISLET IMPLANTATION
(THE PROBLEMS AND THE PROMISE)

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ABSTRACT

The discovery of insulin was a mile stone in the treatment of diabetes. For most diabetics however , the development of the secondary complications of the disease are often fatal and the cause and prevention of these secondary lesions remains the outstanding problem in the treatment of the disease .

Islet cell transplantation as a total cure for the disease was proposed over 80 years ago . Islet implantation in rodents is now a safe and reliable method of reversing the metabolic derangements and preventing diabetic lesions in experimental animals . The problem of isolating sufficient islets from the human pancreas and in avoiding the immune tissue rejection phenomena have so far precluded its successful application in man.

Enzymes of defined activity were used to achieve disaggregation of the human pancreas . A periperfusion system was developed for evaluating the dynamic aspects of insulin release both from normal and dispersed pancreatic tissue . This allowed critical aspects in the technique of tissue disaggregation to be examined and improved ,such that in excess of 60% of the initial islet function could be recovered following disaggregation.

Implantion of this crude pancreatic disaggregate in the canine model resulted in uncontrolled fluctuations in blood glucose levels , a massive bleeding diathesis and hepatic trauma, indicating the need for purer islet preparations .

Attachment of islets to a collagen substrate was found to allow recovery of islets as an essentially pure preparation. Tissue culture techniques were used to confirm the viability of pancreatic islets isolated in this way .

Finally the problem of abrogating the immune tissue rejection phenomena was investigated across a discordant xenogeneic histocompatibility barrier . The immunogenicity of human islets was shown to be altered following tissue culture of 28 days such that survival of 3 months was achieved in an immunocompetant host .

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INTRODUCTION

I can think of no more appropriate introduction to this dissertation than the rather tragic case notes of Prof. Dr. Kussmaul written exactly 110 years ago .

' As I came to this patient about 11 o'clock in the morning I found her lying in bed in the greatest unease . She was in great pain and oppression , throwing herself here and there and begging for help , in great fear of death ' .

He goes on to describe how extensive treatment produced not the slightest improvement in her condition and how the unfortunate woman eventually died in great distress.

This serves to remind us of what a terrible disease diabetes was before the discovery of insulin . It also perhaps explains why in the long history of the disease countless physicians and scientists have directed their endeavours and often their lives in trying to provide an understanding of the cause and treatment of diabetes .

There are literally thousands of names that could and perhaps should be mentioned in a comprehensive review of this subject . Obviously this is impossible within the confines of the limited number of pages allotted to this thesis . Instead an attempt will be made to selectively retrace some of the historical developments which have led to our present understanding of the disease , our endeavours to treat the disease , and the problems which remain unresolved .

CHAPTER ONE

1.1 HISTORY OF DIABETES

Possibly the first recorded reference to diabetes is contained in the Ebers Papyrus purchased by Georg Ebers in Luxor in 1872. This papyrus is one of the oldest known medical documents and is thought to date from around 1,500 years B.C., although Dawson (1965) suggests that it may have been copied from an earlier document dating from around 3,400 B.C.. A complete translation by Joachim appeared in 1890 in which the following mention is made of ' a medicine to drive away the passing of too much urine ';

Branches qadet plant
Grapes
Berries of uan tree

Honey
Sweet beer

cook, filter and take for two days' (Major 1965).

According to Ajgaonkar (1972) many of the symptoms of diabetes i.e. polyuria , glycosuria and the foul breath of the untreated diabetic were known to the Hindu physicians before 400 B.C.

A more definitive description was provided by Aretaeus the Cappadocian (81-138 A.D.). He noted the excessive thirst and urination ,general langour and fatal end point of the disease. He believed that the large fluid intake did not remain in the body but that it 'flowed through and that many parts of the flesh pass out with the urine'. For this reason he likened the effects of the disease to a syphon , the ionic term from which is derived the word diabetes.

Paracelsus (circa. 496-541 A.D.) thought that diabetes was

due to the presence of abnormal salts in the urine and recorded that death resulted when this ' essence settles in the kidneys and salts them ' (Major 1965).

In the 11th Century ,diabetes was extensively described by the Arabian physician Avicenna (980-1037) who noted the abnormal appetite , sweetness of urine , gangrene , and the muscular , mental and sexual impotence (Wellman 1977). He prescribed fenugree , seman contra and hops as a cure . It is interesting that this remedy was repeated by Professor Bobin in Paris in 1913 with some degree of success (Garrison 1966).

Little further was recorded concerning the disease until the Royal Physician Thomas Willis (1621-1675) rediscovered the sweetness of diabetic urine by tasting it , and suggested that the disorder arose first in the blood and then in the urine (Willis 1679).

It is clear from the writings of Dobson (1776) that this finding was still not accepted by many physicians , and it was not until 1787 that William Cullen coined the adjectives 'mellitus' and ' insipidus' to distinguish between the two forms of "diabetes" , (the latter lacking glycosuria).

Dobson (1776) , evaporated the urine of diabetic patients and obtained a brown substance which by appearance and taste was 'indistinguishable from sugar '. He noted (again by taste) the presence of sugar in both the urine and serum of diabetic patients.

Chevruei (1815) the French chemist identified this sugar as glucose. Trommer (1841) introduced a test for glucose in the urine which was followed by the discovery that small

amounts of sugar in urine and blood could be measured by the reduction of copper in an alkali solution (Fehling 1848). This opened the way for a quantitative study of the nature of the disease. Pavy (1862) first recognized that the extent of the glycosuria was related to the level of hyperglycaemia.

Other metabolic defects of the disease were noted i.e. acetone (Petters 1857) , aceto-acetic acid (Gerhardt 1865) and β oxybutyric acid (Stadelmann 1883) were identified in the urine of diabetic patients.

It is known that diabetes was being diagnosed in 1872 by a ' chemical analysis of the urine ' by Prof. Adolf Kussmaul after whom is named the acidosis induced 'air hunger' often present in the terminal stages of this disease (Kussmaul 1874)

It is of considerable interest that the first indication that the pancreas could be associated with the development of diabetes came when Johann Brunner (1653-1727) succeeded in removing the pancreas from a dog and noted the ensuing polyuria and polydypsia. Despite assertions to the contrary (Wellmann 1977) , it appears from the writings of de Sauvages (1752) that Brunner clearly recognized the 'artificial production of diabetes'. It seems that like many experiments in science , these findings associating diabetes with the pancreas were too premature and were not pursued.

Nearly 150 years later Bouchardat (1851) pointed out that diabetics dying from the disease often had a small atrophic pancreas. However these findings were not conclusive for many diabetics had macroscopically normal pancreases.

On March 19th 1853 Claude Bernard presented his zoology dissertation in which he introduced the perhaps then heretic view that animals like plants could synthesize glucose (Unger 1979).He identified the liver as the main site of glycogen deposition and also as the site of glucogenesis , an excess of which was released into the circulation and excreted via the kidneys in diabetic patients.He believed however that this mechanism was under the control of the central nervous system .

In 1883 the experimental work of Arnozan (1884)again implicated the pancreas with diabetes. He showed that pancreatic duct ligation was followed by pancreatic atrophy and subsequent diabetes.

Von Meering and Minkowski (1892,1893) established without doubt ,that the pancreas was related to diabetes. Minkowski after a disagreement with Von Meering (Naunyn 1906) succeeded in removing the pancreas from dogs and showed that , if the dog survived the operation , its urine would contain increasing amounts of sugar and the dog would become severely diabetic. This could be reversed in the short term by reimplanting the pancreas. They went on to show that diabetes did not occur if the pancreas had an intact blood supply and that a neural element was not essential for blood sugar control. In the same year Hedon (1892) noted that in experimental conditions in which the vascular supply to the pancreas remained patent, but in which the external secretion was curtailed ,diabetes did not develop.

Attention was drawn to the possibility that an ' external secretion ' might be instrumental in regulating blood sugar

levels.

In his doctoral thesis Paul Langerhans (1869) had described anatomically isolated clusters of cells scattered throughout the rabbit pancreas , however it was not until 1889 that these structures became known as the ' Islands of Langerhans ' .

Laguesse (1893) suggested that these islands provided the internal secretion instrumental in controlling blood sugar levels.

In 1883 Hoppe-Seyler had drawn attention to the micro-vascular lesions within the pancreas of diabetic patients. Hansemann (1894) reported atrophy of the pancreatic parenchyma and Opie (1901) suggested that progressive fibrosis may damage the islets within the pancreas of diabetic patients. Opie further noted hyalinization and sclerosis of the islands in diabetics dying from the disease. Weichselbaum (1901) described hydropic degeneration or vacuolization and Cecil (1908) noted the hypertrophy and inflammatory lesions (renamed insulinitis by Von Meyenburg in 1940).

Weichselbaum (1901) reported a reduction in both the number and size of islets in diabetic patients . Ssobelew (1902) confirmed these findings and noted the complete absence of islets in 4 out of 13 diabetic pancreases. He further showed that pancreatic duct ligation destroyed all of the pancreas without causing diabetes but that diabetes resulted immediately following the removal of the islets.

Ssobelew concluded that the pancreatic islets produced a substance which played an important if not an exclusive role in the aetiology of diabetes .

Bayliss and Starling (1904) had introduced the concept of chemical messengers for which they proposed the name 'hormone', and De Meyer (1909) concluded from the available evidence that islets produced such a hormone and suggested that it should be called 'insuline'. This hypothesis was independently proposed by Sharpey-Shaffer (1913) who prophetically suggested that it may derive from a substance with a larger molecular weight. The scene was therefore set for attempts to isolate this hormone.

Minkowski (1893), Ssobelew (1902), De Witt (1906), McCallum (1909), Kirkbridge (1912) and Allen (1913) among others are known to have prepared pancreatic extracts. However, the inactivation of any extracted insuline, or the febrile and toxic effects of these preparations precluded their clinical use (Macleod 1920). Zeulzers method (1908) was so encouraging that he signed a contract with Hoechst Pharmaceuticals but no commercial product ever appeared.

The technique of making alcoholic extracts was improved by Scott (1912) and Murlin (1913) whose valuable work was unfortunately interrupted by war service (Garrison 1966).

The Roumanian physiologist Paulesco was probably the first to prepare a suitable extract from duct ligated pancreatic tissue. He showed in 1916 that this extract, if given intravenously, could lower the blood sugar from 140 mg/ 100ml to 26 mg/ 100ml in one hour. Paulesco further noted that this hormone which he named 'pancreine' would also reduce the glycosuria and ketonuria in pancreatectomised animals. However these findings

were not published until July 23rd 1921 (Paulesco 1921).

On the 20th November 1921 , four months after Paulesco's work was published , Banting and Best presented their experimental findings in which they showed that extracts prepared from the pancreatic tissue of glands ligated 10 weeks earlier could reduce the blood sugar if given intravenously or subcutaneously. In May 1922 they published their findings (Banting & Best 1922) on its use in controlling hyperglycaemia in dogs. The alcoholic extraction technique developed by Collip (1923) considerably purified this extract enabling the evaluation of its use in treating the diabetic patient.

There was little doubt that this hormone which Banting and Best christened 'isletine' but which at McCloud's insistence they renamed 'insulin ' (Papaspyros 1964) was the prime agent in reducing hyperglycaemia.

Banting's Nobel Lecture in Stockholm (1925) reported the progress with insulin replacement therapy in the clinical field.

The assumption that the underlying cause of the disease resided solely in a hormone deficiency of the pancreatic islets was however too parsimonious to explain many of the experimental findings. A number of studies indicated that the pathological alterations to the pancreas were neither a constant nor specific finding in diabetes. Hydropic changes and hyalination (Ohlmacher 1904) and vacuolization (Sauerbeck 1904) were demonstrated in the pancreas of non-diabetic individuals. Of more serious concern was the inability to demonstrate any pathological alterations in the pancreases from many known diabetic patients (Sauerbeck 1904), and in many other diabetic subjects doubts were raised as to

whether the observed changes were pathognomic of the diabetic state.

Hence attention became focused upon the actions of several other factors which are now known to induce hyperglycaemia by initiating hepatic glycogenolysis or gluconeogenesis (Bloom 1978).

Hormones of the chromaffin system (thyroid, pituitary and adrenal glands) emerged as real contenders as factors initiating the disease (Falta 1913). Pierre Marie (1886) first described diabetes associated with acromegaly and Minkowski (1887) recorded the presence of eosinophilic adenomas in the disease. Blum (1901) and Zuelzer (1908) both observed hyperglycaemia caused by epiphrine. It is now known that diabetes will occur in about 25% of cases in which hypersecretion of these hormones occur i.e. acromegaly, Cushing's syndrome and pheochromocytoma . Bloom (1978) , however argues that no evidence has emerged to suggest that these hormones induce carbohydrate intolerance unless hypersecretion exists or insulinopenia is already established

An alternative pathogenic theory of the disease was suggested when Allen (1913) managed to prepare a pancreatic extract which when injected into dogs resulted in hyperglycaemia. A similar initial hyperglycaemic effect was observed following insulin replacement therapy by Murlin (1923) who named this as yet unknown hormone glucagon. Glucagon was isolated by Staud (1955) and its presence was identified in the A cells of the pancreas by Baum (1962).

From the work of Diamare (1899) and Mankovski (1902) two

types of pancreatic islet cells (the A and B cells) were known. Since then three other cell types , the C , D and PP cells have been demonstrated (for a review of islet cells see the Lausanne Classification 1977). These cells share certain characteristics and together with certain gut secretory cells are recognized as the APUD (amine precursor uptake and decarboxylation) cells and it is now fashionable in such terms as the gastro-entero-pancreatic (GEP) endocrine system (Orci 1975) or entero-insular axis (Brown 1978).

The work of Muller (1971) and Gerich (1975) indicated that abnormal glucagon secretion in response to both glucose induced hyperglycaemia and insulin induced hypoglycaemia may be a precipitating factor in the disease. This led Unger (1975) to propose his so called 'double trouble theory' and to suggest that deranged glucagon secretion was a prerequisite for many aspects of the disease i.e. the development of ketoacidosis , however this finding has been strongly argued against by Barnes (1976). In addition evidence has been presented by Sherwin (1976) and Shichiri (1982) that glucagon secretion may be apparently normal in diabetics if strict blood glucose control was established with appropriate intravenous insulin infusion systems . This suggests that glucagon is of secondary importance and may exert its effect only during times of insulin deficiency .

A further possibility for the underlying cause of the disease emerged when Feyrter (1966) suggested that cellular secretions may influence adjacent cells i.e. a paracrine relationship existed . Orci (1976) demonstrated the existence of gap junctions between the A and B cells and suggested that

interactions between some cells of the entero-insular axis may play an important part in glucose homeostasis . The increased insulin secretion in response to an oral glucose stimulus as opposed to when it is given intravenously is almost certainly due to pancreatic cell secretion influencing insulin response (Bloom 1978) . It remains possible that a disorder in gut secretions of the GEP endocrine system may play an important part in the pathogenesis or treatment of the disease (Schmidt 1977) but as yet this remains unproven.(for a comprehensive review of this subject see Cruetzfeldt 1980)

The theories of the pathogenesis of the disease presented above emphasize a single causal factor in the development of diabetes , however a major contribution to our understanding of the disease came with the realization that diabetes might be a heterogeneous disease with multi-factorial origins.

In 1908 Naunyn had described three types of diabetes . He distinguished between the 'pure diabetes' i.e. the young diabetic with onset mostly before the 30th year, the 'mild diabetes' usually occurring in older patients and the 'organic diabetes' which was associated with other conditions.

The extensive works of Dr. Joslyn (1921) identified obesity as the greatest risk factor in the 'mild' diabetic .He showed that diabetes was at least 6 times more common in the obese and in certain age groups was 40 times more frequent .

A difference between young and mature diabetics in genetic susceptibility to the disease was reported by Cammidge (1928) and led geneticists to expect that more than one genetic mechanism was concerned in the development of the disease.

However, interest again became focused upon the pancreatic islets when Gellhorn (1941) established the existence of insulin like activity in normal human serum. Groen (1952) developed the rat diaphragm, and Martin (1958) the adipose tissue, in vitro assays for insulin. However Leonard (1959) reported that the insulin like activity measured in human serum was not suppressed by anti-insulin serum raised in guinea pigs, (a serum which completely blocked the biological activity of crystalline insulin). Moreover , insulin like activity persisted in the serum of totally pancreatectomized dogs indicating a non-pancreatic origin for this non-suppressable insulin. It is now known that this hormone is more active in vitro and does not appear to be involved in blood sugar homeostasis (Zapf 1978). Nevertheless it did lead to the realization that normal serum insulin levels were present in many adult onset diabetics (Bornstein 1950). Hence interest became focused upon mechanisms which might inactivate or antagonize the action of insulin in serum. Enzymatic degradation by insulinase (Brohn-Kahn 1949), lipid metabolites (Bornstein 1953) and circulating antagonists i.e. synalbumin (Antoniades 1962) were suggested , however there was no clear evidence to support their importance (Deckert 1965).

Hartcroft (1955) confirmed both normal B cell granulation and extractable insulin from the pancreas of maturity onset diabetics . In contradistinction McClean (1959) indicated that B cells were either absent or their number was severely reduced in the juvenile onset diabetic .

The specific immunological reactive insulin assay developed by Yarlow and Berson (1960) provided an accurate and reproducible technique for the measurement of circulating pancreatic insulin. This method brought about the realization that in the keto-acidotic juvenile onset diabetic, insulin secretion is either totally deficient or severely impaired (Antoniades 1962). These findings were complimented by the extensive studies of Gepts (1965) who showed extensive degranulation, hypertrophy, insulinitis and vacuolization resulting in the almost total disappearance of insulin secreting cells in the pancreas of young diabetics.

In contrast, non destructive islet pathology were often, but not always attendant in the diabetic who developed the disease later in life. The development of diabetes in the face of an apparently adequate insulin reserve was difficult to explain. It has been suggested by Cerasi (1963) that the islets display a delayed or impoverished insulin response to a glucose stimulus. Archer (1975) found that in obese maturity onset diabetes, elevated insulin levels were associated with decreased receptor sites on the cell surfaces in peripheral tissues and Seltzer (1974) showed that the elevated insulin levels were more apparent than real, and were in effect less than those measured in non-diabetic subjects with a corresponding level of hyperglycaemia.

It was clear that real differences existed between juvenile and maturity onset diabetes and that these differences did not reflect solely quantitative gradations of insulinopenia,

indeed diabetes may only be demonstratable under clinical testing conditions or in times of stress i.e. during puberty or pregnancy . In addition other 'types' of diabetes were proposed i.e. the non-ketotic prone 'J' diabetes of malnourished young people (Hugh-Jones 1955) and the inappropriately named maturity onset like diabetes of the young (Tattersall 1974).

Many attempts were made to classify the disease in terms of the degree of glucose intolerance and the age at onset and the diagnostic criteria used to define the disease ,none of which were entirely successful (for a review of classification systems see West 1979).

It was established by Singal (1973) that insulin dependant diabetes was clearly associated with certain haplotypes coded for by the major histocompatibility complex on the 6th chromosome . In particular the presence of HLA-Dr3 and / or the HLA-Dr4 antigens were shown to be common and it was estimated that 93% of insulin dependant diabetics below the age of 20 were either HLA-Dr3 or HLA-DR4 positive (Platz 1981). Immunological factors were further implicated in the disease when Lendrum (1976) established the presence of antibodies to cytoplasmic and membrane components of pancreatic B cells in newly diagnosed juvenile onset diabetics.

The realization that a separate genetic susceptibility and the suggestive evidence that immunological factors may be present in insulin dependant diabetes provided the impetus which resulted in a new classification system and diagnostic criteria being proposed by the National Diabetes Data Group (1979). This classification system was adopted by the World Health Organization

CLASSIFICATION OF DIABETES MELLITUS AND OTHER CATEGORIES OF GLUCOSE INTOLERANCE	
<u>CLASS A.</u>	Diabetes Mellitus.
I.	Type I . Insulin - dependent type (IDDM).
II.	Type II . Non - insulin dependent types (NIDDM).
	a. NIDDM in non-obese.
	b. NIDDM in obese (includes families with autosomal dominant inheritance).
III.	Other types , including diabetes mellitus associated with certain conditions and syndromes .
	1. Pancreatic disease.
	2. Hormonal.
	3. Drug or chemical induced.
	4. Certain genetic syndromes.
	5. Insulin receptor abnormalities.
	6. Other types.
<u>CLASS B</u>	Impaired Glucose Tolerance (IGT)
	a. IGT in non - obese .
	b. IGT in obese.
	c. IGT in association with certain conditions and syndromes .
	1. Pancreatic disease , 2. Hormonal , 3. Drug induced , 4. Insulin receptor abnormalities , 5. Certain genetic syndromes.
<u>CLASS C.</u>	Gestational Diabetes (GDM).
<u>CLASS D.</u>	Previous Abnormality of Glucose Tolerance (Pre. AGT).
<u>CLASS E.</u>	Potential Abnormality of Glucose Tolerance (Pot. AGT).

Table 1

(Tuomilento 1982) and is reproduced in table 1.

This attempt at a historical review of diabetes hopefully demonstrates the important advances which have been made in the understanding of many aspects of the disease however in the vast majority of cases we remain unaware of the underlying cause of the disease process. Thus diabetes remains an 'idiopathic' disease .

It has been suggested that many factors i.e. viral infections (Craighead 1977) , immunological interactions (Cudworth 1978) and toxicity (Prosser 1978) may be instrumental in the onset of the disease but the evidence (except in a few isolated cases) is not persuasive .

If our knowledge of the disease and of factors causing it are incomplete , what of the disease itself ? . As mentioned earlier , death rapidly ensues in the untreated diabetic , but what is the incidence of the disease , and how effectively does insulin ' cure ' it ? .

NATURAL HISTORY

The size or importance of a disease is commonly determined by the number of people who die of it , the number of people who acquire it each year , the number of people who have the disease or the number of people who become disabled because of it.

As Drury (1980) has argued , each of these factors interact and result in imprecise estimates of the size of the problem . For instance , if the mortality from the disease decreases (as has happened) then the number of people with the disease may increase dramatically , indeed the prevalence of diabetes below the age of 40 in America increased 12 fold over the 35 years prior to 1976 (Harris 1982). Similarly , diabetics who live

longer will exhibit a far greater morbidity . In addition West (1975) has reported that the incidence and prevalence of diabetes may vary 4 fold depending upon our definition of the disease. This is illustrated by the study carried out in Birmingham where 0.6% of the subjects were known diabetics but glycosuria was demonstrated in 2.7% of the sample (Malins 1974). One additional factor is that in many studies there are as many subjects who exhibit a 'diabetic' glucose tolerance test but who are nevertheless asymptomatic , as the number of known diabetics in the sample . The significance of this finding awaits greater clarification (Keen 1982).

Despite these reservations there is little doubt that diabetes is an extremely common disease with in excess of 30 million sufferers worldwide (W.H.O. 1980) . Its prevalence is known to vary greatly between countries , populations and sub-populations ranging from an estimated 0.02% of the eskimo population in Alaska (Mouratoff 1967) to in excess of 40% among adult Pima indians (Bennett 1971 , Zimmet 1976). Indeed West (1974) has indicated that if present trends continue a majority of the population in some countries can soon expect to become diabetic .

The W.H.O. Statistics (1980) emphasizes the tremendous health problem which this disease continues to pose. In many countries diabetes is a major cause of mortality and morbidity and in many less affluent societies where insulin is not readily available and health resources are scarce diabetes remains a dreadful and deadly disease. It is erroneous however to think that

this is only a problem of developing countries , (see later).

TREATMENT

Prior to 1922 the only available treatment for diabetes was by starvation therapy or by certain dietary measures.

Dobson (1776) suggested limewater, Rollo (1797) prescribed a meat-only diet and Bouchardet (1875) advised a diet of fat and alcohol. Bouchardet further noted that many diabetics fared better under the prevailing conditions of malnutrition.

Starvation therapy was first proposed by Areteus (81-138 A.D.) and was somewhat successful in increasing the lifespan of the diabetic patient. We know from the extensive data of the Joslyn Clinic that between the years 1897 and 1914 death usually occurred within two years of the onset of the disease. However, during the period 1914 to 1922 when starvation therapy was prescribed the average survival of the diabetic was increased to nearly six years.

While these measures undoubtedly reduced the osmotic diuresis and delayed the onset of other symptoms of the disease , the occurrence of keto-acidosis inevitably proved fatal.

The isolation of Insulin by Banting and Best (1922) and the purification procedure developed by Collip (1923) are now well known and the speed with which this preparation became available is remarkable.

In November 1921 Banting and Best presented their experimental work in dogs from which they concluded that 'the results did not at present justify the therapeutic administration of degenerated gland extracts to patients with diabetes mellitus.' By the time this paper appeared in print, insulin had

been used 7 months previously on a 14 year old boy in Toronto and was being used by a few 'approved specialists'(Sonksen,1977)..

Early experience with insulin was extremely encouraging and on the 19th August 1922 the Times Newspaper of London carried the heading 'Canadian cure for diabetes' and reported that the 15 year old daughter of the United States Secretary for State was in hospital in Toronto undergoing treatment for the disease.

It soon became apparent that the acute metabolic complications of the disease could be prevented by frequent injections of insulin. In addition , the acute medical emergency i.e. diabetic coma (or more accurately , uncontrolled hyperglycaemia, hyperosmolality and keto-acidosis (Alberti 1977)) could , in many cases , be corrected.

Keto-acidotic coma ,which was the cause of death in nearly 66% of diabetic patients prior to 1922 (Marble 1971) , decreased to 15% by 1930 and declined to present day levels by 1940 (Sonksen 1977), being now the cause of death in approximately 1% of the diabetic population (Marks 1971). Nevertheless, it is well to remember that diabetic coma remains a serious condition with a case mortality of between 5 & 15% (Alberti 1977) . It may prove fatal in as many as 50% of elderly patients or when medical assistance is delayed (Gale 1976).

The prolongation of the life of the diabetic being treated with insulin soon became apparent. The mean life expectancy of all diabetic patients at the Joslyn Clinic steadily increased from 4.9 years in 1922 to 18 years in 1970 . This longevity was most marked in those who developed the disease

before the age of 20 , increasing from less than 2 years in 1922 to 27.8 years by 1970 and continues to rise. However, early experience with insulin was not without some problems.

Until 1937 the only commercially available insulin was an amorphous short acting preparation called regular or plain insulin necessitating frequent (sometimes 5 daily) subcutaneous injections to maintain blood sugar levels within acceptable limits..

Frequent localized reactions and lipoatrophy occurred at the injection site (Marble 1971) which are now thought to be mainly due to the unwanted contaminants in these insulin preparations (Teuscher 1974). Impaired growth was common and many children displayed symptoms of Mauriacs syndrome (White 1938) which is now a well recognized complication of poorly controlled diabetes (Guest 1953). These adverse effects almost disappeared following the development of longer acting insulin preparations (Scott 1934) and (Hagedorn 1936) and the availability of purer single peak and mono-component insulins (Gallaway 1971) and the less immunogenic mono-species porcine insulin preparations (Wentworth 1976) .

Montgomery (1979) in a review of insulins and insulin therapy argued that it was possible to select an optimal insulin replacement regime to sustain the diabetic and prevent the acute metabolic effects of the disease. The recent introduction of biosynthetic human insulin produced by recombinant DNA technology provided a useful addition to the wide range of insulins available and is particularly useful in cases of persistent insulin allergy or resistance (Glynne 1982).

Watanabe (1918) first discovered the hypoglycaemic effect of certain guanidine compounds and although their toxicity precluded their general use a similar effect was noted in Sulphonylurea derivatives (Loubatieres 1942) . The introduction of safer forms of sulphonylureas (Franke 1955) and biguanide preparations (Pomeranze 1957) provided a major breakthrough in adult diabetic management. Prior to 1955 it was usual to initiate insulin therapy in all diabetics the effect of which tended to increase obesity and insulin resistance in many already obese diabetic patients (Sonksen 1977). The introduction of these oral hypoglycaemic agents enabled many patients to adequately control their diabetes without insulin and it became apparent that in the mild usually obese diabetic , diet or oral hypoglycaemic agents were usually the best course of treatment (Sherwin 1978).

Thus treatment regimes have evolved to provide adequate control of carbohydrate intolerance and prolong the life of the diabetic . However , this longevity has brought about the realisation of the extent to which major pathological disorders often accompany this disease.

Avicenna (980-1037 A.D.) first noted the susceptibility of diabetics to gangrene of the extremities , Jaeger (1855) described diabetic retinopathy and Rollo (1798) reported peripheral neuropathy but it was not until the 1930's that diabetologists became aware of the magnitude of the prevalence of coronary heart disease , renal failure , blindness , neuropathy and skin disorders in the diabetic patient .

It has been estimated that over 75% of insulin dependant

diabetics will exhibit pathological alterations to the eye within 20 years of the onset of the disease (Caird 1969) . Diabetic retinopathy is now the most common cause of blindness between the ages of 30 and 64 in England and Wales (Kohner 1982). Pirart (1978) in an extensive study reported that 45% of diabetics will develop neuropathy , 55% retinopathy and 15% nephropathy within 20 to 25 years duration of the disease. Gangrene of the leg or foot has been estimated to be between 50 and 70 times more common in age matched diabetics (Bell 1957) , heart disease 20 times more common in adult diabetics (Burton 1980) , perinatal mortality increased 5 times (Larsson 1974) and the risk of dying from cerebro-vascular disease was shown to be almost 4 times greater in the diabetic (Lavy 1973). It is now generally accepted that the occurrence of these major complications are responsible for the early morbidity and mortality associated with the disease.

Marks (1971) has reported that the life expectancy among diabetics of all ages is only 2/3 that of the general population. The mortality statistics of the Joslyn Clinic attribute this increased mortality mostly to large vessel and renal disease and show that this increased risk is most marked in those people who develop the disease early in life. Of the 1,260 diabetics between the ages of 20 and 35 who died between 1956 and 1968 , 78% of the deaths were the result of cardiovascular or renal complications associated with the disease. For the young diabetic retinopathy , coronary heart and renal disease go hand in hand (Balodimos 1971) and there remains a desperate need for treatment which will not only treat the metabolic abnormalities

of diabetes but prevent the dreadful sequelae of complications secondary to the disease.

Despite nearly 50 years of investigation and debate the cause of these secondary complications remains enigmatic and their treatment or prevention constitute the outstanding problem in diabetic research and management today.

Many causes have been proposed . Hypersecretion of growth hormone appears to play at least a permissive role in the development of retinopathy (Lundbaek 1976) and genetic susceptibility to this complication has been proposed on the grounds of a positive chlorpropamide alcohol flush reaction (Leslie 1979) and familial studies (Creutzfeldt 1976). Glucose metabolites i.e. sorbital (Gabbay 1975) have been implicated while it has been suggested that control of hypertension may influence the onset of some lesions (Mogensen 1980). Ristelle (1976) suggests that abnormal collagen synthesis may be influential in renal damage while Brownlee (1979) argues that coronary atheroma may be linked to elevated circulating free fatty acids in the less well controlled diabetic.

Pirart (1978) however argues that the only positive correlate with the development of these secondary lesions is the severity and duration of the disordered blood sugar levels . Not all diabetologists accept this view and consider that the evidence is insufficient to warrant the increased risk of iatrogenic hypoglycaemia arising from more rigid control of blood sugar levels.

Over the past 40 years innumerable clinical studies have

failed to resolve this vital issue. Knowles (1964) after reviewing 300 clinical studies concluded that insufficient evidence existed to prove a mitigating effect of greater control of blood sugar levels on the development of these secondary lesions. The findings of the University Group Diabetes Programme (1970) indicated that neither conventional insulin therapy or treatment with oral hypoglycaemic agents offered any potential in reducing mortality or vascular complications in diabetic patients, indeed they suggested that cardiovascular mortality may even be increased in subjects receiving sulphonylurea and biguanidine compounds. These conclusions have however been severely criticised (Kilo 1979) .

Kaplan (1973) argued that clinical trials comparing varying degrees of often assumed metabolic dysfunction have not and will not provide an answer to this vital question.

There is a wealth of data that similar renal (Federlin 1976), vascular (Engerman 1977) and ocular (Orloff 1978) diabetic lesions can be prevented in experimentally induced diabetic animals following complete restoration of blood sugar control by pancreatic islet cell transplantation. The studies of Mauer (1975) and Federlin (1976) further suggest that regression of existing lesions may occur . Perhaps the most convincing evidence derives from the elegant experiments of Lee (1974) who demonstrated regression of existing diabetic renal lesions following kidney transplantation into isogeneic normoglycaemic rats.

For this reason alone it would appear prudent to strive to achieve the best hormonal and biochemical control possible .

It is now well recognized that conventional insulin therapy

is unable to fully restore the carbohydrate intolerance and metabolic disorders of the disease (Crofford 1975) . In order to achieve this aim insulin infusion systems have been adapted which attempt to mimic normal insulin release in response to changing physiological requirements i.e. during exercise and postprandial and to be responsive to alterations in insulin secretagogues.

Kadish (1965) first described a servoregulated infusion device which delivered insulin in response to changes in measured blood sugar levels (the so called artificial pancreas or closed loop system). Since then improved glucose sensors (Clemens 1977) and algorithms which deliver projected insulin requirements (Kerner 1976) have improved the degree of blood sugar control . Near normal metabolic profiles are now possible (Buckle 1977) at least in the short term . However , these devices are complex and expensive and sufficiently small implantable glucose sensors and miniturized monitoring systems have proved difficult to develop. Shichiri (1982) has reported short term control with implantable glucose sensors in dogs but considerable innovative work is still needed before long term control becomes a reality. One interesting finding of Mirouze (1979) was that extended remission (enabling the complete withdrawal from insulin therapy for periods in excess of four years) occurred in some patients when closed loop systems were used to treat newly diagnosed juvenile diabetics and confirmation of these findings would have important implications in the treatment of diabetes.

More pertinent to the treatment of insulin dependent diabe-

tics have been the portable infusion systems based upon pre-programmed diurnal insulin release models (Irsigler 1979) and long term subcutaneous insulin delivery with postprandial bolus injections (Pickup 1978).

Impressive improvements to a wide range of abnormalities i.e. decreased levels of catecholamines and growth hormones (Tamborlane 1979), ketone bodies (Buckle 1977) and glucagon (Raskin 1979) have been reported. Alberti (1981) , however, reported increased lactate levels in diabetics even when treated with closed loop infusion systems and questions whether metabolism in the diabetic can ever be completely normalized by insulin delivery into the peripheral circulation. In addition it has been shown that diurnal variations in blood sugar levels may be far greater than in non-diabetics although mean blood sugar levels were normal.

The long term effect of these reported improvements in metabolic control remains uncertain and we must wait to see if these improvements are sufficient to abrogate the secondary complications of the disease.

An alternative strategy has been to attempt total pancreatic endocrine replacement by transplantation of the vascularized pancreas or isolated islets of Langerhans.

Early attempts at whole gland transplantation (Kelly 1965) demonstrated that insulin could be withdrawn for a time in all recipients and that normal carbohydrate control could be achieved however mortality was high with less than 20% of patients surviving one year (Lillihai 1970).

Despite improvements in techniques the outcome of whole or segmental grafts remains depressing with an 18% mortality within 3 months and 30% within a year (Sutherland 1983).

To the end of June 1982 there had been 223 whole or segmental grafts reported to the transplant registry. Of the grafts which had been undertaken since 1977 only 10% were still functioning at 1 year with the longest graft surviving 49 months, when unfortunately the patient died from pre-existing diabetic lesions.

It is difficult to draw conclusions from these figures . Most pancreatic transplantation has been carried out in conjunction with simultaneous renal transplantation in patients already experiencing the end stage of the sequelae of diabetic complications. To this must be added the increased risk associated with chronic anti-rejection therapy . Therefore whole or segmental pancreatic transplantation has ,inevitably ,been justified only in those cases where the prognosis is more severe than the risks attendant following transplantation.

At present it would appear that improvements in technique and avoidance of rejection are necessary if whole gland transplantation is to become other than a last final effort in treating the end stages of the disease.

In contrast the initial early attempts at islet transplantation (Sutherland 1978) showed that islet transplantation was safe but ineffective.

To June 1982 there had been 76 cases of allogeneic islet tissue implantation reported to the transplant registry of which only 4 were able to survive without exogenous insulin (Sutherland 1983).However two of these cases (Chasten 1979 and Valente 1980)

were in non-immunosuppressed patients and provide hope that a safe technique for islet cell transplantation is feasible.

The potential benefit of successful islet cell transplantation are clear however its application awaits breakthroughs in the isolation of sufficient islets to fully correct or cure the disease and in controlling the rejection problem.

In conclusion diabetes is a disease of multi-factorial origins . Each new case needs careful appraisal as to the underlying cause and clinical expertise in providing long term treatment and support.

For some , perhaps 20% of insulin dependent diabetics (Colon 1974) and a larger proportion of non-insulin dependent diabetics conventional insulin treatment is capable of providing adequate support to maintain active and useful life into old age but for most insulin-dependent diabetics there has to be a better alternative than the gradual progression towards the debilitating and life threatening sequelae of the disease.

THE CLINICAL PROBLEM

In the United States of America , diabetes is now the 4th. leading cause of death (W.H.O.1980) . The National Diabetes Data Group (1978) has indicated that there are in excess of 5.2 million diagnosed diabetics (2.5% of the population) and that this figure is steadily increasing. The actual figure may be more as Madows (1973) indicated that the survey methods used underestimate by 15% the actual number of diabetics.

There is little doubt that for many of these unfortunate

people the future is depressingly bleak. At least 1.3 million are being treated for coronary heart disease (Rpt. of Nat. Comm. on Diabetes 1975), however there is evidence that coronary heart disease which appears to exert the greatest toll in older NIDDM is declining in line with the decreasing coronary heart disease of the non-diabetic population (Monthly vital stats. rpt.1979). This however is not true for the the ocular and renal lesions which appear to be more frequent in the usually young IDDM , (Knowles 1974) who comprise approximately 22% of the diabetic population (Harris 1982) . There are an estimated 5,000 diabetics who are newly registered as blind each year ; a 25 fold increase over the general population (Rpt. natl. comm . on diabetes 1975) . In addition 3,000 diabetics with irreversible renal damage will either die or in most cases receive maintainance dialysis or renal transplantation (Cameron 1983). This accounts for between 1/4 and 1/3 of the patients accepted for treatment of chronic renal failure in the U.S.A. (Avram 1982) .

I have been unable to find more than one estimate of the financial impact of providing adequate medical care for the diabetic population .Entmacher (1976) estimated that the direct cost of medical services totalled 2.5 billion dollars in 1976 in the U.S.A.To this must be added inflation , the costs of rehabilitation services for blindness and following amputations , the imputed loss of earnings arising from disability and early mortality . The true financial cost must by now be staggering .

The most important aspect of the disease , that is human suffering and the erosion of the quality of life is of

course not quantifiable.

Comparable figures for England and Wales suggest that the percentage of diabetics in the population is smaller than in the U.S.A. . About 600,000 diabetics are known , that is about 1.1% of the population. . Perhaps a more accurate reflection of the size of the problem is that in 1979 , 2,700 hospital beds in England and Wales (1.64 %of the total) were used solely for the treatment of diabetes (D.H.S.S. report MB4 no.13, 1979). This figure is only for those patients who are known to be in hospital for the treatment of diabetes. It does not include hospitalization for the secondary complications of the disease which provide an additional demand upon the medical resources and health care personnel.

It is clear that fiscal policies are already lamentably inadequate to provide help for many of those people who are experiencing the sequelae of secondary complications of the disease.

There are at least 500 diabetics per year who require treatment for end stage renal failure , yet in 1981 only 80 received treatment (Cameron 1983) . Thus at least 84% of diabetics who enter renal failure are allowed to die .

Thus it would appear that diabetes still presents a considerably problem , in terms of suffering and the far reaching social and psychological problems arising from the chronic debilitating effects of the disease.

Finally it is well to emphasize that the ultimate goal must be prevention of the disease. As to when this will become a

reality is unknown . Recent advances in immunology and virology have provided possible avenues of research , however it is felt that prophesizing is best left to the prophets . In the meantime we must strive to provide the best treatment possible.

In this country the aphorism coined by Aretaeus (81-138 A.D.) is particularly appropriate:

' It is impossible to make all of the sick well , for the physician in that case would be superior to the gods: but the physician can secure respite from pain and intervals in disease and can render disease latent '.

This forms the basis for this thesis which attempts to resolve some of the problems in isolating sufficient islets from the human pancreas to restore normal glucose homeostasis , and to investigate manoeuvres which might abrogate the foreign tissue rejection phenomena.

CHAPTER TWO

REVIEW OF ISLET CELL IMPLANTATION

2.1 INTRODUCTION

The duct ligation studies of Ssobelev (1902) clearly demonstrated that it was only the endocrine portion of the pancreas which was necessary to prevent diabetes. He prophesized that islet cell transplantation might eventually provide a cure for the disease.

The idea of replacing only the endocrine portion of the gland and therefore circumventing the problems associated with transplanting the zymogen and enzyme rich exocrine tissue was very appealing . However , the isolation of large numbers of islets dispersed throughout the gland has proved a practical hurdle.

Bensley (1911) was able to hand dissect islets from the guinea pig pancreas . Hellerstrom (1964) refined this micro-dissection technique to isolate islets from the pancreas of the guinea pig and obese and normal mouse in increased numbers. Micro-dissection of islets from the human pancreas has been reported by Havu (1969) and Ferguson (1977). However , the yield (30-40 islets per operator per 45 minutes) has been insufficient for transplantation experiments.

Other approaches have been pursued. Improved islet yield has been reported following progressive exocrine atrophy induced by either duct ligation (Kramp 1975) or by donor pretreatment with an exocrine cell toxin such as DL-ethionine (Payne 1979). Questions have been raised as to whether the induced fibrosis may

also result in metabolic abnormalities of the islets (Bergan, 1977) . In addition it is difficult to see how the application of these or similar manoeuvres can have relevance to the treatment of the diabetic patient .

The question of how to provide the diabetic with a new complement of islet tissue has been approached from three directions ;

1. Implantation of exocrine-poor foetal or neonatal tissue.
2. Transplantation of the enzymatically digested but unseparated adult pancreatic tissue directly into a site which will tolerate the introduction of pancreatic zymogens and enzymes.
3. Transplantation of adult isolated islets freed from the tissue stroma following exposure to enzymatic digestion.

The vast majority of our present knowledge of non-vascularized pancreatic transplantation has been developed and verified in the rodent model. Rodents have proved particularly useful in that transplantation can be attempted using inbred strains and therefore the physiological effect can be evaluated in isograft situations where rejection factors do not play a role. In addition, one of the main problems in islet cell transplantation i.e. obtaining sufficient islets to reverse diabetes can be resolved by using multiple donors.

While it is probable that most of the findings gleaned from rodents can be applied to answering similar questions in man we must remain aware that certain physiological and anatomical differences exist between rodent and large animal models which

may invalidate some of these assumptions.

For this reason it is perhaps prudent to review the experimental rodent work before discussing its application in larger animal models and clinical transplantation.

2.2 THE RODENT MODEL

In 1951 Browning and Resnick (1952) implanted foetal murine pancreatic tissue sub-cutaneously and into the anterior chamber of the eye in alloxan diabetic mice. Reduction of glycosuria and growth of the implants were noted in some animals, but there was no long term effect from the implanted tissue .

The propensity for growth of foetal tissue was emphasized when Chen (1954) noted growth and differentiation of rat pancreatic primordia maintained using standard tissue culture techniques. Coupland (1960) confirmed that the growth potential of foetal rat tissue continued to be expressed following intra-ocular implantation into an acceptable host . He showed that acinar tissue degenerated but that ductal epithelium and endocrine tissue proliferated .

In a long series of experiments House (1958 , 1961) defined many of the factors which influence the outcome of successful foetal implantation. He went on to show that implants grew better in diabetic than in normoglycaemic hosts , however when severely diabetic hosts were used (blood sugar levels in excess of 20 mMol.) no insulin containing cells persisted in the implanted tissue. House (1961) extended these findings and showed that the age of the donor pancreas was critical. The greatest growth occurred when foetal tissue was implanted . Neonatal

tissue harvested 2 - 12 hours after birth showed slightly less growth , neonatal 5-6 day tissue showed negligible growth but persisted while adult tissue disappeared within three weeks.

Since then foetal pancreatic implantation has retained its promise however Moskalewski (1965) provided the main impetus to islet cell research when he showed that the adult guinea pig pancreas could be disaggregated following exposure to the enzyme complex collagenase . Subsequent studies have used this or similar enzyme preparations to obtain islets from the neonatal and adult rodent pancreas. Lacy (1967) showed that islets isolated in this way would exhibit a normal insulin secretory response to alterations in glucose concentrations in vitro. Continued insulin release during culture (Andersson 1976) and the incorporation of radio-labelled amino acids into insulin and pro-insulin have confirmed the viability of islets isolated in this manner (Lin 1973). Collagenase isolated islets now represent the standard preparation with which to investigate their physiological and metabolic function in vitro and their effect in reversing diabetes in transplanted animals.

2.2 a FOETAL AND NEONATAL ISO-IMPLANTATION OF ISLET TISSUE

Embryologically the mammalian pancreas develops from two diverticuli which appear as buds on the primitive gut . Formation of endocrine cells have been demonstrated in the epithelium of these buds and the endocrine cells gradually form into clusters which represent primitive islets.

In the rat , the dorsal lobe of the diverticulum appears midway through the 12th day of intra-uterine life and the ventral lobe appears approximately 12 hours later (Richardson 1977).The

two lobes fuse and embryonic islet like structures appear around the 15th day of gestation. Granulated B cells have been demonstrated after the 15th day of development (Pictet 1972) , however Freie (1975) and Hard (1944) were unable to observe B cells before the 17th day. Insulin is first assayable during the 11th day of gestation (Pictet 1972) and it may be that B cells become functionally active before they attain their structurally recognizable characteristics .

The insulin content of the foetal pancreas increases dramatically between the 15th and 22nd day of gestation , showing an almost three fold increase each day during the last 4 to 6 days of intra-uterine life (Freie 1975 , Hegre 1972). During this period the number of islets more than doubles each day.

Acinar cells and recognizable acini with a few eosinophilic zymogen granules are not recognizable until the 17th day of gestation (Murrell 1966) , after which they increase dramatically during the last 6 days of intra-uterine life (Pictet 1972). Thus foetal and neonatal tissue represents an absolute or relative exocrine deficiency .

It would therefore appear that the age of foetal tissue is critical for successful implantation. Foetal tissue of less than 16 days gestation was ineffective in reversing diabetes when implanted beneath the kidney capsule (Brown 1980) although Usadel (1980) reported growth and survival of both exocrine and endocrine elements in 14-16 day gestation foetal tissue into the dorsal sub-cutaneous space.

When undispersed foetal tissue of over 18 days gestational

age has been used there have been reported cases of cyst formation and tissue erosion ,presumed to be due to the developing acinar tissue, (Mullen 1977 , Brown 1980) .

Brown (1974) was the first to demonstrate that 4 or 6 foetal rat pancreases implanted under the kidney capsule could reverse the diabetic state in 66 % of streptozotocin diabetic rats. Normoglycaemia was restored after 22 days , circulating insulin levels were elevated , glycosuria and polyuria were abolished and a normal glucose tolerance test was obtained.

Hegre (1976) studied the quantitative aspects of implanting foetal pancreatic tissue in to renal sub-capsular sites . He showed that tissue from 4 pancreases would reverse diabetes in 19 weeks but that a similar effect was seen in 11 weeks if 8 pancreases were used. Garvey (1979) and Mullen (1980) among others have subsequently reversed diabetes utilizing the renal sub-capsular site . Other sites which have been used including intra-muscular, intra- testicular and sub cutaneous implantation (Spence 1979) have showed no evidence of graft function. This may be due to the need for early vascularization which Browning (1951) reported to be a prerequisite for survival and growth.

Mullen (1977) suggested that there may be a physiological advantage in diverting the secreted insulin after sub-capsular implantation directly into the portal circulation by performing a reno - portal vein shunt . Spence (1979) found the splenic pulp to be equally effective as a site for reimplantation , however Feldman (1980) was unsuccessful in implanting chopped foetal tissue directly into the portal vein , with only 12% of

experimental rats becoming normoglycaemic and 16% of the animals died from portal hypertension and hepatic failure using tissue from 4 to 8 pancreases .

It is apparent that considerable growth has to occur if amelioration of the diabetic state was to occur . The weight of the pancreas on the 16th day of development is only about 1/600th that of the adult rat pancreas (Hegre 1972) and the insulin content is even more markedly reduced , $0.2 \mu\text{U}$ as against $3,750 \mu\text{U}$ (Brown 1980) .

Brown (1980) went on to show that a single foetal pancreas implanted under the renal capsule of severely diabetic rats would eventually reverse the diabetic state if the rats were maintained on insulin until glycosuria declined. Insulin was then decreased until normoglycaemia was established at an average of 45 days. Brown measured an 800 fold increase in the insulin content of the graft over the 45 day period , eventually reaching 25% of the normal adult value.

Garvey (1979) and Mullen (1980) have emphasized the need for insulin therapy during the period of growth and development of the implanted tissue. However the work of McEvoy (1978) and Mullen (1977) would suggest that a period or degree of hyperglycaemia or insulin deficiency (Halsted's so called 'law of deficiency ' 1909) may provide the greatest impetus to sustained growth of the foetal tissue.

In contrast to foetal tissue , the neonatal pancreas has been shown to comprise approximately 80% acinar and ductal elements and about 20% islet tissue (Matas 1976) , this of

course varies depending upon the age of the neonate .

The first successful reversal of diabetes by neonatal tissue followed intra-peritoneal injection of enzymatically dispersed but unseparated disaggregate from 20 to 35 donor pancreases (Leonard 1973). In the majority of experimental animals , normoglycaemia was restored within a few days. Glycosuria and polyuria was abolished and the rats exhibited a normal intravenous glucose tolerance test (IVGTT) two weeks later.

Other sites of neonatal implantation which have been successfully employed include into the lung (Matas 1977) , abdominal muscle (Axen 1981) and beneath the kidney capsule (Serie 1983).

Matas (1976) showed that the recovery of islet tissue was improved following minimal collagenase digestion. He went on to show that one neonatal pancreas if infused directly into the portal vein could reverse diabetes in 55% of experimental animals. Although it has been suggested that non-endocrine elements may be thrombogenic within the liver only transient increases in transaminase were noted and no long term deleterious effects on liver function were seen (Azamoo 1975) .

Islets become lodged within the portal vein radicals and are rapidly endothelialised within the vascular bed (Matas 1977). Revascularisation of the islets occurs after a mean of 8 days and either through migration or recannalization , move into the hepatic lobules where surprisingly they have been shown to form intercellular junctional complexes with adjacent hepatocytes (Griffith 1977) .

Studies have demonstrated the continued survival of all pancreatic islet cells (Lorenz 1977) within the islets. Mitotic figures have been reported (Leonard 1976) and both islets with normal morphology and chain like regenerating single islet cell lines were observed (Hegre 1976). This would suggest some growth potential and would accord with the findings of Rumpf (1977) who indicated that the full potential of neonatal tissue may take many months to be fully expressed.

It would appear that in distinction to adult islets (Kemp 1973) intraportal implantation of islets from 3 to 4 neonatal pancreases offers no advantage over other sites in terms of the number of animals which become normoglycaemic (Axen 1981). However, direct comparisons between different studies are hazardous in terms of the induced diabetic state and differences between enzymatic dispersal techniques.

Whichever site is used both the number of animals becoming normoglycaemic and the time needed to restore normoglycaemia are directly related to the amount of islet tissue implanted (Leonard 1975).

Both sub-normal (Steffes 1974) and supra-normal (Weber 1976) intra-venous glucose tolerance tests have been reported and would appear to reflect the amount of islet tissue implanted. Indeed, when larger quantities of islet tissue has been implanted, decreased blood sugar levels, hyperinsulinaemia, hyperglucagonaemia and polyphagia have been reported (Weber 1976).

It is clear that if a sufficient quantity of dispersed neonatal tissue is implanted normoglycaemia occurs (Matas 1976)

neonatal tissue is implanted normoglycaemia occurs (Matas 1976) glycosuria is abolished (Leonard 1975) weight gain is restored and other deranged metabolic pathways return to normal (Mauer 1975) .

Concern has been expressed that transplanted rats fail to exhibit the early or 'cephalic' phase of insulin release in response to an oral glucose load (Siegel 1979). This is thought to be due to the lack of islet innervation (Trimble 1980) and/or an increased sensitivity of the implanted islet tissue to catecholamines (Pipeleers 1978) . The long term studies of (Vermitsky 1977) , however have shown that this 'cephalic' phase of insulin release although absent at 11 months had returned to normal by 13 months. It is clear that an impressive restoration of normal glucose homeostasis has been achieved in this model.

One important and as yet unexplained advantage to foetal and neonatal tissue implantation is the finding that in contradistinction to adult islet tissue there have been no reported reversals to the diabetic state of apparently 'cured' animals , (see later)

In summary , the results of foetal and neonatal implantation in the rat model suggest that both represent ideal sources of islet tissue. In the case of foetal tissue it can be transplanted whole without recourse to enzymatic exposure. Acinar tissue is either absent or poorly developed and fails to differentiate following implantation while islet and possibly ductal elements exhibit substantial proliferation potential.

Neonatal tissue may be dispersed and the exocrine

contaminants appear to cause no adverse localized tissue reaction.

In the rat model it has been shown that there are at least four factors which directly influence the outcome of foetal and neonatal transplantation ;

1. The age of the donor tissue.
2. The severity of the animals diabetic state.
3. The amount of tissue implanted.
4. The site of implantation.

It has been shown that under appropriate conditions the endocrine components from one donor pancreas would appear capable of reversing the diabetic state of one adult recipient.

2.2 b ISOGENEIC IMPLANTATION OF ADULT RODENT ISLET TISSUE

In contrast to foetal and neonatal tissue transplantation the implantation of either whole or dispersed but unseparated adult pancreatic tissue has proved unsuccessful (Kostianovsky 1972), and at times hazardous to the recipient. Although reports have appeared indicating that exocrine poor tissue derived from prior duct ligated , or DL-ethionine pre - treated rats may be successfully implanted and that a small degree of exocrine contamination may be well tolerated (Ziegler 1975) , no physiological or morphological evidence of islet function or survival has been obtained following implantation of unseparated adult rodent pancreatic tissue.

For this reason purified or partially purified pancreatic islet preparations have proved most effective in attempts at

iso-implantation in the rodent model.

Moskalewski (1965) showed that at least 70 morphologically intact islets in excess of 150 μ m diameter could be hand harvested from the rodent pancreas following collagenase digestion. Kostianovski (1966) improved the yield of islets by prior disruption of the gland using retrograde injection of fluid into the pancreatic duct. Lacy (1967) used ductal distension and collagenase digestion to isolate islets from the guinea pig, rat and monkey pancreas.

Since then numerous studies have indicated the yield of islets from the rat pancreas could be improved by varying the method of mechanical disaggregation (Matas 1976), the time of enzymatic digestion (Mallaise 1971), the type (Moskalewski 1967) or even batch of collagenase used (Toledo-Pereyra 1977). Other enzymes have partially substituted for, but not as yet replaced collagenase (Henriksson 1977). Sequential digestion procedures (Scharp 1975) and screen filtration (Shibata 1976) to remove the islets freed from the tissue stroma and the digestion solution have been advocated as factors improving the yield of islets. Discontinuous density centrifugation has been used to rapidly separate and harvest islets from collagenase digested tissue, (Scharp 1975). Isolated islets now represent the standard preparation with which to investigate their effect in reversing diabetes in transplanted animals.

Youneszan (1970) was the first to attempt adult islet implantation to reverse alloxan induced diabetes in allogeneic rats. Isolated islets from 4 donors (60% of the normal adult

rat islet tissue) were implanted into the peritoneum , and although significant reductions in glycosuria and hyperglycaemia were noted in the short term , normalization of these parameters did not occur and the islets were almost certainly rejected.

Ballinger (1972) noted a partial although more sustained amelioration of the diabetic state following implantation of 400-600 isologous islets into the peritoneum and Reckard (1973) reported that transplantation of 600-1200 islets into streptozotocin diabetic rats restored the fasting blood sugar levels to normal limits.

Since then successful islet implantation has been reported using a variety of sites. These include implantation into intramuscular sites (Helmke 1975) , implantation into the pancreatic mass and sub-mandibular gland (Georgakakis 1977) , the testis (Ferguson 1973) , the spleen (Koncz 1976) , the peritoneum (Barker 1974) , and beneath the kidney capsule (Reece-Smith 1981) .

Provided a sufficient number of islets are implanted into each of these sites a rapid return to normoglycaemia can be achieved . Other sites such as sub-cutaneous implantation and injection of islets into the systemic circulation have been reported to be ineffective (Barker 1974).

The most advantageous site in terms of the least number of islets needed to provide a curative effect would appear to be directly into the portal vein (Reckard 1978) .

Many investigators have since applied intra-portal implantation of isolated islets to reverse experimentally induced

diabetes in the rat model .

Oakes (1978) was unable to demonstrate any long term deleterious effects on liver function following intra-portal implantation , and although an increased incidence of hepatic cysts (Finch 1977) and tumours (Feldman 1977) has been noted it is most likely that these result from the use of streptozotocin (Lauder 1981).

Implantation of Cr_{51} labelled islets have shown that these remain confined within the liver . Long term quantitative studies of the extractable insulin content of the liver have established that at least 50% (Rabinovitch 1976) and as many as 90% (Trimble 1980) of the islets can survive in this site.

One worrying aspect of adult islet implantation is that many apparently 'cured' animals spontaneously revert to the pre-transplant diabetic state , sometimes after a period of many months. The studies of Rumpf (1977) who implanted large numbers of islets into the peritoneum and portal vein strongly suggest that the recurrence of diabetes resulted from implanting a minimal number of islets to restore the euglycaemic state. Leonard (1975) suggests that this failure was due to exhaustion of the islets by overstimulation .

Thus, isolated islet implantation can represent a safe , effective and reproducible technique for restoring full normoglycaemic control in the rat model . It is clear , however that the effectiveness of islet cell implantation depends upon the site of implantation, the severity of the diabetic state and the number of islets used.

Provided these conditions are met an impressive return to

normal of the metabolic disorders associated with diabetes has been recorded (Lorenz 1975) , (Feldman 1977) .

2.2 c ALLOGENEIC IMPLANTATION OF RODENT PANCREATIC TISSUE

The work of Medawar (1944) established that grafts between genetically different individuals of a species (allografts) were destroyed by a mechanism which was immunological in nature.

It is now known that this allogeneic immune tissue response involves the recognition , proliferation and infiltration by those cells (cytotoxic, helper and suppressor T lymphocytes , B lymphocytes , K cells and macrophages etc.) with specificities for the foreign antigens .

This rejection mechanism is invoked by antigens present on cell surfaces which are encoded for mainly by loci contained within the major histocompatibility complex (MHC) , although less reactive non-MHC histocompatibility loci have been described (Silvers 1977) .

In the rat model the MHC (designated as RT-1) has so far been shown to contain 5 loci . These loci have been further distinguished as those expressing the class I antigens RT-1A and RT-1C , which are expressed on almost all nucleated cells , (Berah 1970) and the class two antigens of which RT-1B (also designated Ia) is the most characterized and are expressed on the surface of only a limited range of cells including macrophages , lymphocytes , dendritic cells (Puri 1980) and in some species on vascular endothelium (Hancock 1982) .

It is clear that when major differences exist in the RT-1 between donor and recipient rat strains , rejection of the

implanted tissue occurs very rapidly.

Slater (1976) and Franklin (1979) have recorded lymphoreticular cell infiltration of the graft within two days followed by progressive lymphocyte and plasma cell involvement and destruction of the engrafted tissue. Similarly, physiological evidence of graft survival (defined as a return to the diabetic state following successful islet implantation) has been reported as having a mean survival time of 3.2 (Nash 1977), 3.7 (Reckard 1980), 4.0 (Morris 1980) and 5.2 (Lacy 1979) days.

It was initially believed that foetal tissue may be less immunogenic than adult pancreatic tissue (Gonet 1965). Most studies however indicate that a rapid immune destruction of the graft occurs in the rodent model (Garvey 1979, Mullen 1980). Spence (1979) reported lymphocyte infiltration of the graft within 4 days and the destruction of the foetal pancreatic allograft before they developed sufficiently to reverse the diabetic state. Mullen (1980) used morphological and physiological criteria to investigate allogeneic foetal implantation between various strains of rats and reported that the longest mean survival time was less than 10 days. One report exists however of long term allogeneic foetal islet survival in the dorsal sub-cutaneous space in non-immunosuppressed outbred rats (Obando 1975). It may be that this is an immunologically privileged site (Barker 1977) for foetal pancreatic tissue. It is interesting that Shumakov (1980) reported survival of human foetal pancreas in this site in immune competent rats.

The work of Strautz (1970) and Gates (1972) suggested

that allogeneic islet survival and function could be maintained by encasing the islets within a Millipore chamber of $0.45\mu\text{M}$ pore size . Other investigators have however been unable to reproduce these results (Helmke 1975) , (Buscard 1975) , Tze (1979) and Garvey (1980) . A recent study (Helmke 1982) suggests that these synthetic chambers , even allowing for islet survival, are insufficiently permeable to insulin diffusion to restore physiological carbohydrate control . In addition problems of fibrotic reactions around the capsule would appear to preclude their long term use .

Most attempts to prolong allogeneic islet cell survival have involved the use of non-specific immunosuppression , i.e. immunosuppressive agents which are known to act by depressing the immune system . Pharmacological agents which have proved effective in averting rejection following clinical organ transplantation have however proved less successful in prolonging islet allograft survival in the rodent model .

Neither Azathioprine (Finch 1976) , (Vialettes 1979) , or Prednisolone (Nelken 1977)) have prolonged the fate of allogeneic islets by more than a few days at the most .

Cyclophosphamide has been reported to prolong allogeneic islet survival in some studies (Finch 1977) , but other studies have reported this agent to be ineffective (Bell 1980) .

The relatively new immunosuppressive agent Cyclosporin A has also proved ineffective in prolonging graft survival when recommended levels of the drug have been administered (Morris 1980) , (Rynasiewicz 1980) . Reports to the contrary exist ,

(Violettes 1979) , (Reece-Smith 1983) and (Squifflet 1983) , but in these cases , what are now thought to be near toxic levels of the drug were administered (Cameron 1983) .

One immunosuppressive agent which has almost universally been shown to have a beneficial effect on allogeneic islet survival has been the use of anti-lymphocyte or anti-thymocyte serum or more accurately the gamma globulin component of that serum. The functional survival of islets transplanted across a strong histocompatibility barrier has been prolonged from between 4 to 7 days to in excess of 60 days in many studies (Finch 1977) , (Lacy 1979) , (Reemstma 1981) .

Two further regimes of non-specific immunosuppression are of note. The use of intra-venous silica , a macrophage immobilizing agent , has been used to prolong islet allograft survival to in excess of 100 days in 80% of recipients (Nash 1980) . Total lymphoid irradiation TLI has been carried out by some investigators . In these cases TLI prior to engraftment in conjunction with other forms of immunosuppression has led to life long acceptance of allogeneic islet grafts . The reported high mortality and incidence of tumours (mostly fibrosarcomas) following exposure to the high levels of radiation used (Mullen 1983) suggests that this approach may prove hazardous in man.

In general non-specific immunosuppression has proved ineffective in prolonging adult allogeneic islet grafts . In addition the attendant risks of infection and neoplasms suggest that this approach may be inappropriate in the clinical field .

Similar results have been obtained in the few studies

attempting allogeneic foetal and neonatal transplantation (Mullen 1982), . To date only one non-specific immunosuppressive regime has proved effective in prolonging allogeneic foetal grafts when Mullen (1980) reported life term immunological unresponsiveness following TLI and either donor bone marrow or foetal liver infusion.

The initial work by Murray (1935) , who implanted pancreatic tissue following culture in the recipients serum introduced the first attempt at specific immunosuppression of islet tissue. This may be conveniently viewed as the selective suppression of the donor antigen presenting cells thought to be responsible for the induction of the allogeneic tissue rejection phenomena. Specific immunosuppression may be either antigen induced (active enhancement) by the introduction of preparations containing donor histocompatibility antigens or antibody induced (passive enhancement) by treatment of the recipient with antibodies directed against donor antigens .

The realization that not all antigens need to be 'covered' by enhancing antibodies (Fabre 1974) , and that incompatibilities within the RT1.A , RT1.B and RT1.C loci differ not only in the type of immune response they elicit (Klempnauer 1983) but also in their ability to invoke the cytotoxic T cell response (Klein 1977) have important implications in the field of pancreatic transplantation.

Although the mechanisms proposed differ , it has been suggested by Bach (1976) , Wagner (1976) and Lafferty (1977) that the allogeneic T cell response may be induced by

costimulatory activity (stimulator cells) arising from 'recognition' of the RT1.B (Ia) antigens on the surface of implanted cells .

Whatever the validity of this proposal , it has been shown that passive enhancement by antibodies directed against the donor RT1.B antigens results in the long term survival of islet allografts in the murine model (Faustman 1981) .Recent studies using RT1.B antibodies in the rat model reported only modest prolongation of islet survival (Reece-Smith 1983).The reason for this discrepancy remains unclear but it is possible that the differences may be due to the specificities of the antibodies employed .

Active enhancement has been attempted by injecting donor strain foetal liver (Nelken 1977) or spleen cells (Smith 1983) prior to transplantation . The level of induced tolerance in these experiments has been highly varied but surviving allografts at 157 to 200 days was reported in 3 out of 14 rats (Smith 1983)..

An alternative approach , i.e. by altering the immunogenicity of the donor islets has been attempted by some investigators.It was first promulgated by Summerlin (1973) and shown by Boyles (1975) that pancreatic islets , following a critical period in culture , may be effective in achieving long term allogeneic islet survival in rats. Boyles (1975) reported that implantation of allogeneic adult islets , cultured for 14 to 16 days in 95 % oxygen restored normoglycaemia for between 3 and 6 months in 80 % of experimental animals . Two rats remained normoglycaemic for 11 and 12 months.

It is believed that islet cells do not express RT1.B (Ia) antigens (Baekkeskov 1981) and that the adherent and lymphoreticular cells which are thought to initiate the immune response are either lost or lose their immunogenicity during the period of culture.

Bowen (1980) was able to prolong islet survival in non-immunosuppressed mice in excess of 3 months following culture for 7 days in high oxygen levels . Lacy (1979) reported survival in excess of 100 days following culture of hand picked islets for 7 days at 24 C combined with a single injection of ALS. Reetsma (1981) was unable to reproduce these results and suggested that a contributory factor may have been his inability to identify and exclude small lymph nodes from the islet preparation .

These findings provide convincing evidence that allogeneic implantation into a non-immunosuppressed recipient may be possible. However , the optimal conditions for preservation of islet tissue and for the loss of tissue immunogenicity remain undefined . Lafferty (1983) indicates the necessity of high oxygen levels , however Yasunami (1983) reported rat to mouse xenogeneic islet survival following 7 days culture under atmospheric oxygen levels . Low temperature culture combined with ALS immunosuppression (Lacy 1980) and culture of rat mega-islets in 95 % oxygen (reported by Bowen (1980) to prevent islet disintegration in high oxygen levels), have been applied successfully in prolonging xenograft survival (Lacy 1982).

Both Mandel (1979) and Garvey (1980) reported that they

were unable to reduce the immunogenicity of foetal pancreatic tissue by in vitro culture techniques. Extended culture for 17 days before even minimal prolongation of islet tissue survival was reported by Simeonovic (1981) , who recorded that the rodent foetal pancreas is grossly contaminated with lymphoid tissue which may explain this lack of success.

More encouraging results were recently reported by Collier (1983) when following organ culture for 23 days , 7 out of 25 allogeneic foetal pancreatic grafts survived at least 104 days, at which time 4 of these animals were normoglycaemic .

2.3 ISLET CELL IMPLANTATION IN LARGER ANIMALS

Early attempts to restore normoglycaemia by islet implantation in larger animals were unsuccessful .

Sutherland (1974) implanted a semi-purified islet preparation into the peritoneum and thigh muscle in pigs with no evidence of graft function . Kemp (1973) reported immediate death in dogs following auto-implantation of islet tissue into the liver. Kumar (1973) reported progressive atrophy of implanted canine foetal tissue into the anterior chamber of the eye in dogs.

The first indication that implantation of islet tissue in larger animals might prove successful was provided by Scharp (1975) . Following 70% resection of the monkey pancreas and the administration of 50 mg/kg streptozotocin , followed by collagenase digestion and ficoll separation of the resected tissue , the islets were implanted into the portal vein. Two of the four experimental animals were reported to be normoglycaemic

at 3 weeks following implantation and one animal remained normoglycaemic for the 6 week term of this study. An IVGTT however showed only a marginal improvement over the diabetic state of the control animals .

Similar results were reported by Matas (1976) and Lorenz (1979) in partially pancreatectomised and streptozotocin induced diabetic dogs .However , the use of this experimental design must be questioned.

In these circumstances it is difficult to determine the relative contribution of the residual pancreas and implanted tissue in restoring carbohydrate control in this experimental design . This is particularly important in view of the finding (Trimble 1977) that implantation of islet tissue can result in the recovery or promote the growth of insulin containing cells within the residual pancreas. While the technical difficulties in ensuring the completeness of pancreatectomy and the high mortality resulting from this procedure in rats (Slepjevic 1975) justifies the use of a chemically induced diabetic but not insulinopenic state . In larger animals the completeness of surgical resection can be ensured and would appear to be more appropriate.

The most impressive studies so far of islet cell implantation were those carried out by Mirkovitch (1976) . He implanted unseparated collagenase digested pancreatic tissue from the horizontal portion of the gland directly into the splenic pulp of pancreatectomised dogs following cannulation through the splenic vein. All experimental animals became normoglycaemic within 10 days and no significant difference was noted in IVGTT's carried out in transplanted and normal animals during the

ten week term of the experiment. Similar results have recently been reported by Madureira (1982) . In both studies splenectomy resulted in diabetes and death confirming the role of the intra-splenic tissue in maintaining normoglycaemia.

The effectiveness of intra-splenic auto-implantation of digested tissue derived from a portion of the canine gland has been confirmed in other studies (Kolb 1977) and (Orsetti 1981) . Normoglycaemia was established in these cases but an IVGTT was reported as grossly abnormal .

In some studies the entire canine pancreas has been enzymatically disaggregated and implanted into the splenic pulp Kretschmer (1977) , (Mehigan 1980) , Horaguchi (1981) and Long (1983) . In these studies between 88 and 100 % of transplanted animals became normoglycaemic . One study reported a normal IVGTT in 3 out of 4 dogs (Long 1983) .

Several factors have been shown to directly influence the outcome in this model of intra-splenic canine implantation .

A critical time for exposure to enzymatic digestion appears to exist (Kretschmer 1979) . Dispersed tissue implanted before this time failed to restore normoglycaemia (Kretschmer 1977) although the studies of Gray (1979) suggest that finely chopped but undigested pancreatic tissue may be well tolerated and showed evidence of continued insulin secretion . Prolonged exposure to the enzymatic digestion leads to a progressive loss of insulin containing tissue (Kretschmer 1979) .

At this stage of digestion most investigators have reported that isolated islets are not readily seen , but remain entrapped

within the non-endocrine tissue stroma (Mirkovitch 1976).

The survival of both endocrine and exocrine tissue components have been reported within the spleen , however it would appear that exocrine tissue does not maintain its secretory function (Hadji - Georgopoulos 1982). It is clear that intra-splenic implantation can be effective and although increased serum transaminase levels have been reported (Kolb 1977) suggesting a deleterious effect upon liver function these however , returned to normal within 4 weeks.

In contrast infusion of a portion (Matas 1976) or the total enzymatically dispersed canine gland (Kretschmer 1977), (Mehigan 1980) , (Horaguchi 1981) and (Dobroschke 1981) into the portal vein has been less successful , although Kolb (1977) and Madureira (1982) reported no difference between the two sites.

The development of portal hypertension following portal implantation in dogs (Matas 1976) has been a consistent finding with the development of a fatal haematological dysfunction resembling disseminated intravenous coagulation occurring in all animals in the studies of Mehigan (1980) and Dobroschke (1982).

Evidence of extensive thrombosis of the portal system and massive internal bleeding was seen at autopsy. It has been suggested that anticoagulation therapy could prevent these haematological changes . The study of Mehigan (1980) tends to support this view as anticoagulation decreased the severity of these disorders , but deaths have still occurred in fully heparinised animals (Mehigan 1980) and (Marner 1982).

In those studies where intra-portal implantation has been

successfully carried out most reporters have shown that the percentage of animals which become normoglycaemic is much less than when implantation is carried out into the splenic pulp (Horaguchi 1981).

Three attempts at auto- implantating islet tissue in larger species (other than man) have been reported .

Hoverstein (1977) was unsuccessful in restoring normoglycaemia in pancreatectomised monkeys by intra-splenic or intra-peritoneal auto-implantation of dispersed pancreatic tissue Mieney (1979) reported success in autoimplanting dispersed pancreatic tissue into the portal vein in baboons . In 2 out of 4 experimental animals , blood sugar levels within the normal range were reported during the 6 week term of the experiment . Marner (1982) was unsuccessful in restoring normoglycaemia following implantation into the portal vein in pigs . Islet cells were demonstrable at autopsy , however their function as measured by C peptide secretion was insufficient to reverse the diabetic state.

2.3 b ALLOGENEIC IMPLANTATION IN LARGER ANIMALS

The limited number of studies dealing with allogeneic implantation in the dog suggest that the findings are similar to those reported in the rodent model .

The immune destruction of implanted pancreatic tissue in non-immunosuppressed canine recipients has been reported to occur within 3 (Kolb 1979) , 5 (Toledo-Pereyra 1982) and 8.5 (Zammit 1979) days. A limited number of immunosuppressive regimes have been attempted . To date neither Azothioprine and

Prednisilone (Kolb 1979) , (Kretschner 1979) , Cyclosporin (Du Toit 1982) or ALS (Toledo-Pereyra 1982) have proved unsuccessful in prolonging graft survival by more than 10 days.

Whether specific immunosuppression or long term culture will be more effective remains unknown.

One further report exists of an unsuccessful study in implanting foetal into maternal and foetal into sibling pancreatic tissue in monkeys (Jonasson 1977).

2.4 HUMAN PANCREATIC ISLET TISSUE IMPLANTATION

Several early attempts at implanting either foetal or neoplastic islet tissue in man have appeared in the literature. Intra-muscular and sub-cutaneous pockets were among the first sites chosen .

Cultured islet apudoma cells were implanted into the axillary tissue of a diabetic patient by Murray (1935) and into two diabetic patients by Porter (1956) . Although a slight reduction in requirements was reported by Murray he did not consider this significant. Urca (1970) implanted an insulinoma beneath the fascia lata of a young insulin resistant diabetic . Insulin containing cells were shown to be present 3 months later but complete fibrosis of the graft had occurred at 9 months. No physiological evidence of graft function was obtained in this patient and his insulin requirements were not significantly altered.

Implantation of foetal tissue was attempted by Craig in 1941 (reported Craig 1968) into the axilla of three diabetic patients , however all grafts failed . Brooks (1959) was also

HUMAN ISLET CELL IMPLANTATION REPORTED IN THE

LITERATURE UP TO DECEMBER 1983

Principle Reporter and Centre	Allografts			Autografts
	Adult	Infant	Foetal	
SUTHERLAND (Minnesota)	12	6	0	10
LORENZ (Berlin)	7	0	0	0
VALENTE (Genova)	14	0	13	14
TRAVERSO (Los Angeles)	0	0	0	4
CAMERON (Baltimore)	0	0	0	8
DOBROSKA (Gießen)	0	1	0	4
REEMTSMA (New York)	0	0	0	1
NARDI (Boston)	0	0	0	5
HINSHAW (California)	0	0	0	5
GROTH (Stockholm)	0	0	9	0
LARGIADER (Zurich)	6	1	0	0
GRODINSKI (Detroit)	0	0	0	3
WILSON (Boston)	0	0	0	1
NARASIMHAM (Minneapolis)	0	0	1	0
CHASTAN (Bordeaux)	0	0	1	0
USADEL (Frankfurt)	0	0	1	0
TOLEDO-PEREYRA (Detroit)	0	0	0	6
Totals	32	8	25	68

Table 2

unsuccessful in significantly reducing the insulin requirements of diabetic patients following foetal implantation into muscle pockets .

The main surge in studies involving islet implantation began in 1977 when Sutherland (1978) began a series experiments using allografts derived from adult and infant pancreatic tissue . Since then there have been 133 attempts at islet cell implantation either published or reported to the International Islet Transplant Registry (Sutherland 1980) , (table 2) .

To date, 40 attempts at allo-implantation of adult or infant derived pancreatic tissue have been carried out (table 3). In addition , foetal tissue from either a single or multiple donors have been implanted in a further 25 patients (table 4).

The techniques of tissue preparation have varied considerably. In most ,but not all cases ,collagenase digestion has been used to disaggregate adult and infant pancreatic tissue. Some investigators have attempted a partial purification of the islets , while others have implanted unseparated tissue disaggregates. Mechanically disaggregated foetal tissue has been implanted without recourse to enzymatic digestion.

2.4a ALLOIMPLANTATION OF PARTIALLY PURIFIED ISLET PREPARATIONS

Although there have been no studies in man comparable to isolated islet implantation in rodents , some investigators have attempted to purify islets but the final preparation still represents a grossly contaminated preparation .

Sutherland (1978) attempted to purify islets by density centrifugation using the sucrose polymer Ficoll. He implanted

Principle Reporter and Centre	Number of Recipients	Period of IDDM (yrs.)	Donor Tissue	Islet Yield Islet Volume	Attempts to Purify Islets	Site of Implantation	Evidence of Graft Function
Largader	4	28 - 35	Adult	50 to 300,000	No	Portal Vein	None
	2	29 - 38	Adult	50 to 300,000	No	Splenic Pulp	None
	1	23	Infant	200,000	No	Splenic Pulp	Decreasing insulin for 9 months. Insulin free from 9 until 19 months following implantation.
Sutherland	3	Long standing	Adult	0.2 to 0.4 %	Density Centrifugation	Intra-peritoneal	Slight reduction in insulin requirements in 2 cases.
	1	Long standing	Adult	14.5 %	Density Centrifugation	Intra-muscular	None
	4	Long standing	Infant	0.4 to 12 %	No	Intra-peritoneal	Reduced insulin requirements in 2 patients
	2	Long standing	Infant	8.5 to 9 %	No	Intra-peritoneal	Reduced insulin requirements in one case
	8	12 - 42	Adult	20 - 60 %	No	Intra-portal	Sustained insulin reduction in one patient only.
	13	13 - 36	Adult	5 to 90,000	Culture	Sub-cutaneous in Millipore Chambers	2 Patients insulin independent at 3 months, one of which remained insulin free at 22 months.
Valente	1	-	Adult		Culture	Intra-peritoneal in Millipore Chambers	Presumably none.
DOBROSHKE	1	32	Infant	-	No	Intra-splenic	No insulin reduction at 1 month.

Table 3

IMPLANTATION OF ALLOGENEIC FOETAL PANCREATIC TISSUE

Principle Author and Centre	Period of IDDM	Number of Recipients	Number of Foetal Donors Used	Foetal Size	Storage of Tissue	Site of Implantation	Immuno- suppression	Evidence of Graft Function
GROTH (Stockholm)	14 & 31 years	1	6	13-30 cm	Cultured 3hrs-15days	Intra-portal	Yes	Rising C-peptide levels for 4 months to 5% of normal.
	'Long standing'	1	15	13-54 cm	Cultured 1 - 4 days	Intra-portal	Yes	None at 5 months
	'Long standing'	1	15	13-54 cm	Cold Storage ½ 3 hrs.	Intra-splenic	Yes	None at 2 months
	'Long standing'	6	1	13-54 cm	None	Intra-muscular	2 Yes 4 No	None None
VALENTE (Genova)	16 & 18 years	2	4 & 5	10-15 wks	Cultured	Intra-muscular	None	Increased C-peptide levels but no significant decrease in insulin requirements
	12 & 15 years	2	4	11-15 wks	Cultured	Intra-portal	None	No change in insulin requirement in 1 .Insulin reduced from 80 to 20 Units per day in other case.
	9 - 20 years	9	1 - 3	10-22 wks	None	Intra-muscular	None	None in 6 patients .2 cases had an insulin reduction at 6 months 1 insulin free at 6 months.
USADEL (Frankfurt)	None.recent pancreatectomy	1	1	20 wks	Cultured	Intra-muscular	Yes	None
CHASTON (Bordeaux)	8 months	1	60 fragments	9 wks	Cryo- preserved	Intra-muscular	None	Insulin free after 62 days . Became insulin dependent after 15 months.
MARASHIMAM (Minneapolis)	Over 20 years	1	1	19 wks	Cultured	Intra-portal Intra-muscular	None	Insulin requirements reduced by 66 % at one month.

Table 4

partially purified collagenase digested adult pancreatic islet tissue into the peritoneum in 3 patients. One further patient received separated islet tissue into an intramuscular site . In each of the three intraperitoneal trials the amount of islet tissue recovered was extremely low (less than 0.4% of the normal human islet mass) and although a transient decrease in insulin requirements occurred in one patient no long term effects of the implanted tissue were noted. The intra-muscular recipient received a larger volume of tissue (14.5% of the adult islet mass) but no metabolic effects were seen.

An alternative approach to islet purification was carried out by Valente (1982) . During short term culture techniques , it has been reported that islet tissue selectively survives while exocrine tissue becomes non-viable (Matas 1976). Valente cultured semi-purified islet preparations for several days and attempted to avoid the immune rejection phenomena by implanting these islets encapsulated in millipore chambers as allografts in a series of 14 diabetic patients. Between 5 and 90 thousand encapsulated islets were engrafted sub-cutaneously in 13 recipients and into the peritoneal cavity in 1 patient. In 13 patients insulin requirements were diminished in the 3 months following implantation , but as in animal studies (Helmke 1982) this was not maintained , presumably due to fibrotic reactions which Valente found to encircle the bio-artificial chambers. Nevertheless it was reported that two patients were able to be completely withdrawn from exogenous insulin therapy for 3 months and one patient remained insulin independent at 1 year.

In these studies the amount of islet tissue remaining following digestion , and more importantly the purification techniques used , was usually extremely low . In many cases one would not have expected the recipients metabolic state to be greatly influenced from this small volume of islet tissue.

The demonstration that digested but unseparated pancreatic tissue containing an increased quantity of islet tissue was apparently well tolerated in the canine model prompted its application in clinical trials.

2.4b ALLOIMPLANTATION OF UNSEPARATED DISPERSED PANCREATIC TISSUE

To date ,dispersed pancreas implantation of allogeneic adult tissue has been performed in 14 cases and alloimplantation of dispersed infant tissue in 8 cases .

Sutherland (1980) implanted collagenase digested unseparated pancreatic disaggregates into the portal vein of 7 diabetic patients (one patient received two implants). No adverse reaction to the implanted tissue was reported with the exception of the patient who underwent two implants and in whom transient but severe portal hypertension (60 cm water) occurred. The islet yield following digestion , as estimated from the dispersed tissue insulin content , ranged from 20% to 60% of normal adult values. Two patients were temporarily withdrawn from insulin therapy between the 2nd and 4th and the 13th and 17th post-operative days indicating that some early islet cell function may have resulted. Increases in urinary C-peptide levels were measured in two patients for one month following implantation . However , in the other five cases no evidence of graft function was

obtained . No patient was permanently withdrawn from insulin therapy and no long term decrease in daily insulin requirements were apparent.

Largiader (1980) attempted allo-implantation of dispersed pancreatic tissue into the mesenteric vein in 4 patients and directly into the spleen in two patients . No evidence of graft function was noted.

2.4c ALLO-IMPLANTATION OF UNSEPARATED INFANT PANCREATIC TISSUE

Sutherland (1980) implanted collagenase digested unseparated pancreatic tissue derived from a single infant donor into 6 patients with diabetes of 9 - 35 years standing. In two patients islet tissue equivalent to 8.5% and 9 % of the normal adult islet mass was implanted into the peritoneum. In one patient the daily insulin requirements were temporarily reduced from 80 to 28 Units per day three to five weeks following implantation. In four patients dispersed infant tissue containing between 0.4% and 12 % of the adult islet mass was implanted into the portal vein . In three cases diminished insulin requirements were noted between^e the 2nd and 9th weeks following implantation, however all patients have subsequently returned to their pre-transplant insulin requirements . Paradoxically , the patient who received the least amount of islet tissue was able to decrease his daily insulin requirements from 72 to 32 Units after 7 weeks and continued to be adequately maintained on this reduced dose at 18 months.

Largiader (1980) implanted dispersed pancreatic tissue containing about 200,000 islets from a 2 year old child into the spleen following sub-capsular puncture . The recipient was a 23

year old female who developed IDDM 12 years previously. Daily insulin requirements decreased until insulin therapy could be discontinued completely 8 months following implantation. This patient remained insulin independent until for some unknown reason the graft failed after 19 months.

In the one other case of allo-implantation of infant tissue, Dobroschke (1982) implanted dispersed pancreas derived from a newborn child with congenital nesidioblastosis into the spleen of an immunosuppressed patient with IDDM of 32 years duration. A decreased insulin requirement was reported at 14 days post-op but no further details on this patient have as yet been reported.

2.4d ALLO-IMPLANTATION OF FOETAL TISSUE

Valente (1982) has reported on the implantation of foetal pancreatic tissue into 13 non-immunosuppressed patients with IDDM of between 9 and 13 years standing. Cultured foetal tissue from 4 or 5 donors were implanted intra-muscularly in two patients and into the portal vein in two patients. No change in insulin requirements were noted in three of these patients, however in one patient receiving intra-portal foetal tissue the daily insulin requirements declined from 80 Units a day prior to implantation to 20 Units per day at 9 months. Nine patients received minced foetal pancreases into the abdominal rectus muscle. One to three foetal donors were used for each recipient. Six patients showed no change in insulin requirements while two patients showed a slight reduction. One patient was able to be withdrawn from insulin at six months and remained so at one year.

Groth (1980) implanted a single foetal pancreas into the abdominal rectus muscle of two immunosuppressed and 4 non-immunosuppressed patients . No evidence of graft function was obtained in any of these patients. In a further three immunosuppressed patients multiple donors were used. In two patients 15 foetal pancreas , following storage at 4°C for up to 3 hours were implanted into the spleen in one case and intraportally in the other recipient .There was no evidence of graft function at 2 and 5 months. In the remaining case 6 cultured foetal pancreases were implanted into the portal vein. One month later urinary C-peptide levels were recorded and increased to 5% of normal adult values by 4 months ,but then ceased. It is possible that immune reactions were involved in this graft failure for islet cell surface antibodies were detected which coincided with the cessation of urinary C-peptide secretion.

Three other attempts at foetal implantation have been reported .

Usadel (1982) implanted a single foetal pancreas following 8 days in culture into the brachioradialis muscle of a 29 year old female who had undergone total pancreatectomy. No evidence of graft function was obtained.

The work of Narasimham (1977) is interesting in that he attempted to reduce the immunogenicity of a single 19 week foetal pancreas by culturing a dispersed cell suspension from the gland in co-culture with cells from the recipients bone marrow and lymph nodes . Skin sensitization studies following injection of 100 islet cells beneath the skin of the left forearm induced no signs of rejection i.e. erythema . The remaining tissue was implanted

into the liver and intra-muscularly and the patient was reported as being maintained on one third of his pre-transplant insulin requirements one month later .No further details on this interesting study have been reported however .

Chaston (1980) cryopreserved 30 relatively undifferentiated foetal pancreas of 9 weeks gestational age . He implanted 60 fragments of approximately 1 mm³ into the pectorialis muscle of a 25 year old diabetic diagnosed as insulin dependent 8 months previously.This patient became insulin free 62 days later and remained so for the next 11 months . Blood sugar levels then became erratic and it became necessary to reintroduce insulin therapy 14 months after foetal implantation .

In summary , it is extremely difficult to evaluate the technical and immunological aspects of alloimplantation at this time . In most cases , graft function , at least sufficient to allow withdrawal from exogenous insulin therapy has not occurred. Moreover evaluation of the degree of restored pancreatic function is often obscured by differences in the pre-existing diabetic state .For instance,in some patients with diabetes of long standing , C-peptide levels were demonstrable , indicating some measure of residual insulin secretion (Valente 1982) ,(Chaston 1980). Nevertheless , there is indirect evidence that human islet cell implantation has in many cases provided islet cell function (Valente 1982) and in one case at least been successful in restoring full metabolic control .

In those clinical studies where the implanted tissue has provided no evidence of or has ceased to function it is not

possible to determine whether this failure arises from technical or immunological factors.

Although the results of Groth (1980) suggest that immune responses may have been involved in the cessation of function of one foetal graft it is by no means clear that this represents allo-geneic tissue rejection mechanisms. Islet cell surface antibodies are a well recognized finding often at the onset of the disease and an equally plausible explanation is that recurrent disease may have occurred .

It is clear that foetal implantation has been successful in restoring normoglycaemia in non-immunosuppressed patients for in excess of one year which must raise the question of whether human islet foetal tissue is as immunogenic as evidence in the rodent model would suggest. The available evidence suggests that foetal tissue may prove a safe and efficient technique for reversing the metabolic disorders of diabetes but that currently very little information concerning the critical aspects of successful foetal implantation is available.

The effect of rejection mechanisms on graft failure has been somewhat clarified by clinical experience with islet cell autoimplantation in patients undergoing partial or total pancreatectomy in cases of chronic pancreatitis (see later).

Evidence from vascularized whole gland transplantation strongly suggests that the adult pancreas is subject to normal allogeneic immune tissue rejection mechanisms and it may be expected that many graft failures may have occurred for this reason.

AUTO-IMPLANTATION OF ADULT PANCREATIC TISSUE FOLLOWING TOTAL OR PARTIAL RESECTION

Principle Author and Centre	Period of IDDM	Number of Recipients	Number of Foetal Donors Used	Foetal Size Foetal Age	Storage of Tissue	Site of Implantation	Immuno- suppression	Evidence of Graft Function
GROTH (Stockholm)	14 & 31 years	1	6	13-30 cm	Cultured 3hrs-15days	Intra-portal	Yes	Rising C-peptide levels for 4 months to 5% of normal.
	'Long standing'	1	15	13-54 cm	Cultured 1 - 4 days	Intra-portal	Yes	None at 5 months
	'Long standing'	1	15	13-54 cm	Cold Storage ½ 3 hrs.	Intra-splenic	Yes	None at 2 months
	'Long standing'	6	1	13-54 cm	None	Intra-muscular	2 Yes 4 No	None None
VALENTES (Genova)	16 & 18 years	2	4 & 5	10-15 wks	Cultured	Intra-muscular	None	Increased C-peptide levels but no significant decrease in insulin requirements
	12 & 15 years	2	4	11-15 wks	Cultured	Intra-portal	None	No change in insulin requirement in 1. Insulin reduced from 80 to 20 Units per day in other case.
	9 - 20 years	9	1 - 3	10-22 wks	None	Intra-muscular	None	None in 6 patients .2 cases had an insulin reduction at 6 months 1 insulin free at 6 months.
USADEL (Frankfurt)	None, recent pancreatectomy	1	1	20 wks	Cultured	Intra-muscular	Yes	None
CHASTON (Bordeaux)	8 months	1	60 fragments	9 wks	Cryo- preserved	Intra-muscular	None	Insulin free after 62 days . Became insulin dependent after 15 months.
MARASHIMAM (Minneapolis)	Over 20 years	1	1	19 wks	Cultured	Intra-portal Intra-muscular	None	Insulin requirements reduced by 66 % at one month.

Table 5

2.5 AUTO-IMPLANTATION OF THE ADULT HUMAN PANCREAS

Although there are many surgical procedures available for the treatment of chronic pancreatitis, partial resection (Child 1969) and pancreatico-duodenectomy (Braasch 1978) are the two commonly employed. Both methods have their proponents . However, diabetes is a predictable outcome following total , and is the usual but not inevitable outcome following major resection of the gland (Mehigan 1980) .

There have been 68 attempts at implanting autologous dispersed pancreatic preparations following total or partial resection of the pancreas of which 54 have been reported in detail (table 5) .

2.5a AUTO-IMPLANTATION FOLLOWING TOTAL RESECTION

Dobroschke (1982) attempted to purify islets by repeated washing and centrifugation of the collagenase digested tissue. He estimated that between 70 and 100 thousand islets were implanted into the portal vein in 3 patients following total resection , and in 1 patient following partial resection of the gland . All 4 patients required insulin and were adequately maintained on ~ between 10 and 44 Units per day . In one patient who underwent total pancreatectomy endogenous C-peptide was demonstrated, possibly indicating that some islet function was restored.

Lorenz (1981) has reported on 4 of 7 autografts carried out following total resection of the gland . The collagenase dispersed pancreatic tissue containing between 15 and 25 % of the normal pancreatic insulin content was infused slowly into the portal vein. He recorded no significant increase in liver enzyme release or decrease in liver function following implantation ,

but in one patient elevation of portal vein pressure precluded the implantation of more than 80 % of the dispersed tissue. Three patients were subsequently insulin dependent and are maintained on 8,10 and 12 Units of insulin per day . The remaining patient was insulin free with an essentially normal IVGTT two months later. In this patient serum C-peptide levels were 30 % of the pre-operative levels.

Traverso (1980) performed total pancreatectomy and intra-portal implantation of collagenase digested pancreatic tissue in four patients . In two cases only the portion of the pancreas to the left of the portal vein was used while in the other two cases the whole pancreas was processed . Portal hypertension and systemic hypotension developed in 3 of the recipients so that only 1/3 to 1/2 of the dispersed tissue could be implanted. Serum transaminase levels were 20 times the normal level following implantation suggesting some cellular damage within the liver. These returned to normal by the 7th post-operative day. All four patients have required insulin and were being adequately maintained on between 15 and 32 Units per day.

2.5c AUTO-IMPLANTATION FOLLOWING PARTIAL PANCREATECTOMY

Hinshaw (1981),disagregated the pancreas by handprocessing the chopped ,partially digested (4 cases) and non - digested tissue (1 case)through a metal sieve in a series of autografts following partial resection of the pancreas . Following partial purification by sedimentation and centrifugation the final tissue volume implanted into the portal vein was approximately 10 cc . This contained between 500,000

and 2,000,000 islets and islet fragments . It is unclear what this represents in terms of islet volume. Two of the patients remain normoglycaemic with normal glucose tolerance tests , one patient was insulin free for 20 months and then required insulin therapy and two patients have remained insulin dependent.

Short term culture of dispersed pancreatic tissue in an attempt to purify pancreatic islets has been attempted by Reemstma (1980) , who implanted minced but not digested adult pancreatic tissue following 7 days in culture into a subcutaneous space in the forearm of a patient who became diabetic following a partial pancreatectomy .No evidence of graft function was noted.

Valente (1982) reported implanting partially purified pancreatic disaggregate as autografts following 93 % to 97 % resection of the gland for pancreatitis. Approximately 150,000 islets , following 7 days in culture, were implanted intramuscularly in one patient with no evidence of graft function. In three other patients 25 to 320 thousand islets , cultured for between 2 and 4 days ,were implanted into the peritoneal cavity. Two patients remained insulin independent while in the remaining patient , glucose control was being controlled with hypoglycaemic agents. IVGTT's performed in these patients following implantation showed K values between 0.8 and 1.3 .

In a further series of ten patients Valente (1982) implanted collagenase digested , unseparated pancreatic tissue containing between 25 and 400 thousand islets into the portal vein following 93 - 98 % resection of the pancreas .He reported that no adverse reactions to the implanted tissue occurred but it was noted that

portal vein pressures remained elevated from their pre-operative levels in all patients 7 days later. Nine patients became normoglycaemic by the end of the first week. The remaining patient was reported to require insulin 3 weeks following implantation (Valente 1980) but subsequently required no exogenous insulin (Valente 1982). In this series of patients IVGTT's performed between 1 and 12 months following implantation were essentially normal with K values between 1.1 and 1.9. Cannulation of the suprahepatic and portal vein showed a slight increase in blood insulin levels indicating intra-hepatic secretion from the implanted tissue, but whether this increase was sufficient to fully account for the excellent control of glucose homeostasis must remain speculative.

Cameron (1981) reported on 8 patients who underwent 95% pancreatectomy and implantation into the portal vein of unseparated disaggregate. Between 38 and 83 ml of sedimented tissue was implanted. Increased portal vein pressure of up to 60 cm water occurred in all patients following implantation. Normoglycaemia was established in a mean of 28 days in 6 of these patients. Three of these patients reverted to the diabetic state at 3, 6 and 8 months following implantation and three remained insulin independent at 9, 18 and 22 months. In the three patients who remained normoglycaemic Cameron obtained evidence of markedly increased insulin and glucagon levels in blood samples withdrawn simultaneously from the portal and supra-hepatic vein. Of the remaining two patients one had a massive intra and post-operative bleeding diathesis accompanied

by disseminated intravascular coagulation , requiring multiple transfusions of blood and blood products . This experience prompted this group to infuse aprotinin and heparin with the disaggregated tissue , a procedure which was shown to modulate haematological disorders following pancreatic implantation in the canine model. Despite this measure one further patient developed severe portal hypertension which at laparotomy 24 hours later was found to compromise the venous drainage of the large and small bowel, and a meso-caval shunt was constructed . This patient died 10 days later.

Toledo-Pereyra (1983) reported on 6 cases where islet autoimplantation was carried out following 80 to 95 % resection of the pancreas. Two of these patients remain insulin independent one of which exhibited an essentially normal oral glucose test three weeks following implantation . Two patients were reported to be insulin free immediately following implantation are now being maintained on 20 and 83 Units of insulin per day . The remaining two patients died . One case has been reported in detail by Mittal (1980) in which disseminated intravenous coagulation and massive bleeding followed the implantation of pancreatic digest . In the remaining patient massive bleeding also occurred (estimated to be in excess of 10 litres) following implantation with pulmonary vascular congestion occurring after surgery . Problems associated with sepsis , internal bleeding and a perforated transverse colon necessitated further surgery and the patient died 3 months later from multiple causes .

Grodinski (1981) has performed intra-portal implantation of unseparated tissue in 3 patients following 90 % resection of the

gland . In one patient portal hypertension from 15 to 45 cm water persisted for four hours following implantation . All three patients have required insulin therapy and catheterization of the suprahepatic veins demonstrated no significant portal insulin secretion.

Two other groups have carried out (but not reported) islet cell implantation. Wilson (reported by Lorenz 1982) implanted cultured pancreatic tissue into the peritoneal membranes . This patient remained insulin dependent. Nardi (reported by Sutherland 1980) implanted dispersed tissue following 95 % resection of the gland into the portal vein in two and sub-cutaneously in one patient. All three patients require insulin. In a further two patients Nardi implanted dispersed tissue into the portal vein following 80 % resection of the gland. Both patients remain normoglycaemic.

2.6 CONCLUSIONS

In experiments in animals, techniques have emerged enabling the successful reversal of chemically induced diabetes following implantation of isolated islets and neonatal and foetal pancreatic tissue . In addition in some (but not all) hands, implantation of dispersed pancreatic microfragments has been effective in larger animals.

In these animal models critical factors have been identified which directly influence the outcome of implantation . There is also evidence that under certain conditions long term survival of allogeneic and even xenogeneic islet grafts in non-immunosuppressed recipients may be obtained.

Whether these conditions apply in the human model remains unclear. Alloimplantation of adult , infant and foetal pancreatic tissue preparations into insulin dependent patients has in most cases failed to ameliorate the diabetic state. Yet , allo-implantation of foetal (Chaston 1980), (Valente 1982) and infant pancreatic tissue (Kolb 1980) have allowed insulin therapy to be withdrawn for many months in three insulin dependent diabetics two of whom received no immunosuppressive treatment.

Despite these encouraging findings and although experimental allo-implantation in animals remains an intense area of research , clinical attempts are now very infrequent and only 3 have been attempted between 1980 and 1983 (Sutherland 1983).

Continuing experience with auto-implantation would suggest that the main reason for this is the inability to reproduce in man the effectiveness of techniques developed in experimental animals .

In discussing islet cell implantation in man , Largiader (1977) described the procedures used as safe but ineffective.

Time has shown that these assertions may no longer be tenable.

Total or partial resection of the pancreas is carried out in an endeavour to cure intractable pain resulting from chronic pancreatitis. The question of whether to perform total or partial resection must remain a matter of clinical judgement and persuasion , however it is only following total pancreatectomy that unequivocal evaluation of the degree of restored islet cell

function can be made.

There have been 12 cases of islet cell implantation following total resection of the pancreas. One patient remains normoglycaemic with an essentially normal IVGTT. Most of the remaining 11 patients are being maintained on fairly low daily insulin requirements, which provides suggestive evidence that some graft function may have been restored. Direct evidence that this is so has been provided in some of these patients.

Sufficient evidence has now emerged following implantation of both total and partially resected glands to indicate that the procedures used can involve considerable risk to the recipient. Four deaths have been reported following implantation of dispersed microfragments, of which three were directly attributable to the procedures used. In other cases portal hypertension have been common and cardiac dysrhythmia, disseminated intravascular coagulation and systemic hypotension have presented serious risks.

It is clear that a desperate need still exists for a technique of islet preparation which can be shown to be safe to apply and in addition to provide a reproducible treatment enabling total endocrine replacement therapy to be carried out as a cure for the many insulin dependent diabetic patients.

CHAPTER THREE

DETAILS OF PROPOSED RESEARCH

3.1 INTRODUCTION

This chapter describes the rationale behind the experimental work carried out over a 4 year period.

Where help has been provided this is gratefully acknowledged at the end of this thesis , but essentially the experimental rationale to be described and its practical application were designed and carried out solely by the author .

3.2 EXPERIMENTAL DESIGN

Our initial experience suggested that procedures which presented few problems with the rodent model were inappropriate when dealing with the pancreas of larger animals and man. The human pancreas proved resistant to ductal distention , it was extremely difficult to chop into small pieces with either scissors or scalpel blades ,and perhaps more importantly enzymatic disaggregation proved rapid and uncontrolled towards the end of the digestion period. Cell viability in these preparations was invariably very low although small numbers of islets were obtained . It was apparent that considerable improvements in these techniques were necessary if sufficient islets were to be available to ensure adequate carbohydrate control following implantation into an insulin dependent diabetic .

With this in mind it was decided to investigate aspects of the 'rodent' technique of islet cell isolation in an attempt to identify where the procedures could be specifically improved .

The initial steps necessary for rodent islet isolation are now fairly standard . They consist of prior disruption of the

gland , mechanical or hand chopping of the tissue and enzymatic digestion. It is perhaps appropriate to briefly consider each in detail .

Prior Disruption

The purpose of distention of the gland is to initially disrupt the tissue permitting subsequent enzymatic digestion to release islets from the tissue stroma. In the rat , injection of fluid into the pancreatic duct provides distention and disruption of the tubulo-acinar endpieces and the desired acinar-endocrine separation. However, ductal distention of larger animals and the human pancreas is ineffective in this respect (Scharp 1980).

It has been shown in dogs (Downing 1979) that a more satisfactory way to initially disrupt the pancreatic parenchyma was by injection of fluid into the pancreatic vein.

In dogs , each islet receives arterial blood from a single afferent arteriole , and exits via multiple efferent arterioles into the acinar portal circulation (Fujita 1976). It has been suggested that the afferent and efferent arterioles are separated by sphincters (Wharton 1934) which Downing (1979) suggests allows tissue disruption while at the same time preserving the integrity of the pancreatic islets.

A similar islet-acinar portal circulation has been described in the pancreas of other animals (Fujita 1976). Whether venous distention of the gland proves as effective in the human model however awaits clarification .

Mechanical Dissociation

It is usual to cut the tissue sample into small pieces in order to aid enzymatic digestion and to prevent necrosis of the

central tissue during the period of digestion.

Because of the large volume and fibrous nature of the human pancreas , it is extremely difficult to chop into sufficiently small pieces with either scissors or razor blades . Weber (1977) reported that even relatively small volumes of human pancreas i.e. biopsy specimens required up to 30 minutes to chop.

A variety of mechanical chopping aids have been described which will reduce the entire gland into sufficiently small fragments within a few minutes. These chopping aids include rows of parallel razor blades set 1mm apart (Gunnarsekaran 1979), electrically driven counter rotating blades (Gray 1977) , tissue homogenizers (MacDonald 1980) and modified kitchen implements (Gordon 1981).

Mehigan (1981) has suggested that the method of mechanical chopping is not important . Yet the few studies which have attempted to critically evaluate the effect of these devices upon cell recovery and survival would argue against this assumption. Matas (1976) estimated in rats that mechanical chopping resulted in a 39 % loss , while Kretschmer (1977) using the canine pancreas showed a 45% loss in the insulin content following mechanical chopping of the tissue .Chick (1979) reported that a counter rotating blade , similar to that used by Gray (1977), resulted in unacceptably high cellular damage when used to process the pancreas from larger animals and man.

It would appear that if cellular damage is to be minimized then considerable care is necessary in the choice and evaluation of a particular mechanical chopping aid .

Enzymatic Digestion

Since the initial finding more than 60 years ago by Rous (1916) that exposure to trypsin could disperse mammalian cells, enzymatic digestion is now the usual procedure in effecting tissue disaggregation.

Weymouth (1974) has provided an excellent review of the use of various enzymes and other agents which have proved effective in disaggregating a wide variety of tissues.

It is generally agreed that the choice of enzyme will depend upon the age and nature of the tissue to be dissociated. When dealing with a relatively fibrous tissue such as the pancreas, intercellular adhesion can be most effectively reduced by enzymatic treatment of the fibrous tissue stroma. Thus, perhaps not surprisingly, collagenase or collagenase containing mixtures have proved the universal agent used in attempts to effect primary tissue dissociation of the human pancreas.

Commercial preparations of collagenase are available from many sources and are obtained following the ammonium sulfate precipitation of extracellular filtrate of various strains of *Clostridia histolyticum*. In addition to pure collagenases i.e. enzymes capable of hydrolyzing collagen under physiological conditions of pH and temperature, these preparations have been shown to contain a number of other enzymes with proteolytic activity (Mandl 1971). Not all commercial enzyme preparations have proved equally effective in their ability to release viable islets or islet cells (Moskalewski 1965).

Inconsistencies between batches of collagenase in their efficiency in isolating islets have been reported (Toledo-Pereyra 1979). Neilson (1980) has observed that a reduced

insulin response of isolated islets could be traced to a specific batch of collagenase. Cytotoxic 'collagenase' preparations have even been described (Moskalewski 1969) . There is little doubt that many of the reported deleterious effects of these enzymes can be traced to impurities within the commercial enzyme preparations . Enzyme contaminants of collagenase have been shown to have a deleterious effect upon cell survival (Moskalewski 1974) , cell ultrastructure (Amsterdam 1978) and upon membrane receptor sites (Selawry 1978 , Gingerich 1979). In addition it has been reported that a low molecular weight contaminant of commercial collagenase may induce irreversible stimulation of the cellular adenylate cyclase system (Lacombe 1979) , thus resulting in permanent activation of cAMP activity .

It is felt that the question of whether purer or alternative enzymes preparations can increase islet recovery and viability deserves special consideration .

It remains unknown whether purified collagenase alone is capable of dissociating pancreatic tissue , or whether as Sutherland (1981) implies , these contaminants play an essential role in tissue breakup. If the former is true then there appears to be little reason for using a variable enzyme complex , contaminants of which may have as yet unknown effects upon the islet yield and function .

One further and to some extent related aspect of enzymatic disaggregation techniques is the need to establish the end point of the digestion process, i.e. the time when the greatest number of islets can be isolated. Islets isolated after this time may be subjected to damage due to enzymatic overdigestion , while

underdigestion results in islets remaining entrapped within the non-endocrine tissue stroma (Scharp 1980). It is possible that the identification of this end point of digestion may prove less critical if a less cytotoxic enzyme preparation is used to effect tissue disaggregation .

Assessment of Islet Function

Finally , it is necessary to formulate the criteria to be applied in determining whether a particular procedure is truly effective in producing a sufficient quantity of viable islet tissue to restore normal carbohydrate metabolism.

It is clear from a review of the human clinical trials that two procedures have emerged for reporting the yield of recovered human islet tissue.

In many cases , the number of islets have been estimated by direct counting of islets in an aliquot of pancreatic digest (Valente 1982) . It has been suggested that the number of islets may be related to the total number of islets in the human pancreas and thus provide a measure of islet recovery ,however this is open to two serious sources of error.

According to morphological studies in man (Clark 1913), direct counting methods following intra-vital staining have shown that the number of islets vary considerably between individuals .In addition , there is still no general agreement on the absolute number of islets in the normal adult human pancreas and most estimates range between 200,000 and 2,000,000 (for a review of islet numbers and volume see Volk 1977) .

There is agreement however that the size of islets in the human pancreas is highly varied . Ogilvie (1937) estimated that

the weight of individual islets ranged from 0.48 to 2.74 μ g . Tenjings (1947) has provided a mathematical computation of the size classification . This accords with the studies of Hellman (1959) who reported that the number of islets increased as the diameter of islets decreased , indicating a preponderance of smaller size islets. The bulk of the insulin containing tissue was however contained within islets belonging to the medium size classification. Thus, determination of the number of islets recovered must be accompanied by the size classification or insulin content to provide a meaningful estimate of the recovered islet mass .

An alternative method used to estimate the islet yield has been to assay the insulin content of the dispersed pancreatic tissue . Providing that insulin granulation is complete , the extractable insulin content of the pancreas has been shown by Leonard (1973) to provide an approximation of the islet tissue volume . This technique has provided valuable information on the remaining insulin containing tissue following digestion and where islet loss may occur in the separation procedure.

Both approaches , namely islet numbers and insulin content , suffer from the disadvantage that they provide ,at best ,only a quantitative evaluation of the remaining islet tissue and tell us little of the tissue viability .

In some instances , dye exclusion tests of viability have been used , but the inaccuracy of this method has been long recognized (Phillips 1973) . In addition it is clear that inaccuracies may be compounded when dye exclusion is used to estimate the viability of islets where the tissue fragments may

be many cells deep.

It may be argued that , as in animal experiments , in vivo assessment of human islet function may be obtained following implantation . This is not entirely persuasive when dealing with human tissue for the risks of implantation cannot be quantified unless an adequate islet function can be assured. The development of an in vitro method of evaluating islet function is therefore an essential part of evaluating the separation technique .

In some studies , small numbers of human islets , have been tested by batch incubation (Andersson 1976) and periperfusion procedures (Ferguson 1977) . Periperfusion techniques provide the advantage that both quantitative and qualitative measures of the capacity of individual islets to release insulin in response to various secretagogues may be obtained. These have not yet been successfully applied to estimating the IRI release from human chopped pancreatic tissue or in evaluating the remaining endocrine function of the pancreatic disaggregate .

There is a desperate need for a system capable of providing reproducible estimates of the rate of insulin release both from normal and disaggregated pancreatic tissue over a given period of time and in response to insulin secretagogues. Only then will it be possible to critically evaluate and improve the procedures detailed above , and ensure that sufficient viable islets are available for implantation .

Implantation

Once the procedures are established and a sufficient quantity of dispersed islet tissue is available it remains to be shown whether (1) implantation of the unseparated i.e disaggre-

gated pancreatic tissue or separated i.e. isolated islet preparations will restore normal carbohydrate control and (2) whether either can be safely infused without for example the development of portal hypertension or severe haemodynamic changes (Cameron 1981).

Evaluation of the effect of implanting pancreatic tissue must of course be first carried out in an animal model .

Islet Separation

Even if implantation of unseparated dispersed pancreas is successful there are valid physiological (Dobroschke 1982) and immunological (Lacy 1982) reasons why the implantation of a relatively pure endocrine preparation is to be preferred .

To date , attempts to purify human islet tissue by either centrifugation or sedimentation have resulted in low yields of islet recovery (Sutherland 1979) , (Dobroschke 1982) . It is intended to investigate alternative techniques for purifying islet preparations and evaluating their survival under tissue culture conditions .

Materials and Methods

The materials used have been presented in Appendix I . Appendix II contains the methods used . Preparation of media and soluble collagen is presented in Appendix III. The periperfusion design is detailed in Appendix IV . Fractionation and purification of commercial collagenase has been published in abridged form (Compton 1982) but is presented in full in Appendix V .

CHAPTER FOUR

RESULTS

Before assessment of the techniques used to effect islet isolation can be meaningful it is necessary to first establish control values of the various parameters to be measured using adult human pancreatic tissue .

It is unknown how the period of cold storage may influence these results and therefore we have excluded data on those glands with cold ischaemic times in excess of 4 hours .

4.1 STUDIES ON NORMAL ADULT HUMAN PANCREATIC TISSUE

The insulin ,total protein and amylase content was estimated on whole or near whole adult human pancreas , (table 6).

These results indicate that there was a wide fluctuation in the total insulin content of individual pancreas , ranging from 25,000 to 535,000 ng of insulin per g tissue . In one instance we measured the insulin content of the pancreas from a child who died following the onset of diabetic coma . In this case the insulin content was found to be 950 ng/g tissue or only 0.06 % of the average normal value .

One point of note is that the mean insulin content (157,500 ng /g tissue) differed greatly from the median (82,500 ng /g tissue) suggesting that the distribution may not be normally distributed . Filliben's test for normality (corr. coeff. 0.89) and Grubb's test for outliers ($T = 0.87$ and 2.49) indicated that the data was failing to fulfill the criteria for normality for good reasons and not because of outlying extreme values .

TISSUE COMPONENTS OF THE NORMAL HUMAN PANCREAS

	Insulin ng g wet wt.	Total Protein mg g wet wt.	Amylase mg g wet wt.
	31,280	208.9	10.67
	360,460	173.1	2.3
	120,380	147.6	2.62
	54,190	149.1	2.78
	84,440	167.6	4.56
	52,780	157.2	4.35
	513,820	110.6	2.09
	83,030	161.7	3.23
	175,830	172.3	10.66
	241,152	90.9	11.13
	535,300	108.3	7.28
	400,040	148.7	5.05
	25,800	98.9	7.79
	91,538	114.6	4.64
	240,360	106.9	5.39
	91,080	182.6	4.67
	73,905	198.2	4.53
	121,340	110.7	3.42
	69,380	142.1	4.35
	25,990	124.3	11.2
	223,740		
	81,350		
	57,308		
	25,382		
mean	157,500	143.7	5.63
median	82,500		

table 6

Because it has been reported (Dickson 1977) that the recovery of insulin from tissue extracts may be at times low and variable and to ensure that these differences did not arise from inaccuracies in the method of extracting and measuring tissue insulin a recovery experiment was carried out .

Duplicate samples of a known weight of pancreatic tissue from the same pancreas were taken to one of which a measured amount of insulin was added. It was assumed that any inaccuracy in estimating tissue insulin content would be reflected by an

RECOVERY OF ADDED INSULIN FROM SPIKED
SPLIT SAMPLES OF PANCREAS

Exp. No.	Measured Insulin in Homogenate ng	Insulin Added ng	Expected Recovery ng	Actual Recovery ng	Percentage Recovery
H02/80	14,550	32,000	46,550	39,050	83.9
H02/80	31,920	8,000	39,920	27,300	68.4
H02/80	11,700	8,000	19,700	33,600	170.6
H02/80	8,800	16,000	24,800	33,600	135.5
H03/80	11,100	8,000	19,100	22,000	115.2
H05/80	9,200	16,000	25,200	25,030	99.3
H06/80	23,340	320,000	340,000	132,800	39.1
H06/80	20,320	160,000	180,320	194,000	107.6
H06/80	37,120	160,000	197,120	63,700	32.3
H06/80	60,410	80,000	140,410	101,640	72.4
H06/80	48,190	80,000	128,190	127,020	99.1
H07/80	203,630	160,000	363,630	231,120	63.6
H07/80	194,400	80,000	274,400	330,240	120.4
H07/80	158,570	160,000	318,570	331,320	104.0
H07/80	192,860	320,000	512,860	483,360	94.3
H13/80	519,200	32,000	551,200	515,400	93.5
H13/80	255,960	16,000	271,960	400,680	147.3
H13/80	269,640	16,000	285,640	515,900	180.6
H13/80	407,360	6,400	413,760	504,000	121.8
H16/80	14,260	none	14,260	14,700	103.1
H16/80	18,300	25,600	43,900	67,600	154.0
H16/80	5,400	19,200	24,600	15,000	61.0
H16/80	1,960	12,800	14,660	34,880	237.9
H16/80	5,320	5,320	11,720	22,560	192.5
H17/80	64,800	6,400	71,600	72,600	101.4
H17/80	61,600	3,200	64,800	126,040	194.5
H17/80	53,380	3,200	56,580	88,550	136.7
H17/80	97,980	none	97,980	110,400	112.7
H101/81	41,150	800	41,950	39,270	93.6
H101/81	79,000	400	79,400	99,040	124.7
H101/81	73,140	16,000	89,140	93,280	104.7
H101/81	5,520	16,000	21,520	22,125	102.8
H104/81	41,180	320,000	361,180	191,700	53.1
H104/81	70,680	320,000	390,680	232,960	59.6
H104/81	18,630	8,000	26,530	36,850	138.4
H104/81	20,680	800	21,480	23,500	109.4
H104/81	33,520	16,000	49,520	45,100	91.1
H106/81	13,540	6,400	19,940	9,325	46.8
H106/81	12,920	8,000	20,920	27,030	129.2
Mean	82,337	63,244	145,455	140,622	

TABLE 7

increase or decrease in the expected insulin content (tissue insulin content of the duplicate sample plus the added insulin) when compared to the spiked sample. This recovery experiment was carried out on 40 duplicate samples (Table 7) .

The mean insulin content of the non-spiked samples was 82,337 ng to which was added an average of 63,244 ng of exogenous insulin so that the expected recovery of insulin (assuming no insulin loss occurred) was 145,455 ng.

The measured insulin content of the spiked samples averaged 140,622 ng which indicates a recovery of tissue insulin of 96.7%.

The variance between individual estimates is illustrated in fig 1 , which shows the log insulin content of expected against actual recovery. Linear regression analysis confirmed the accuracy of the method over the range measured ($K = 0.93$) with negligible loss of insulin during the extraction and measuring.

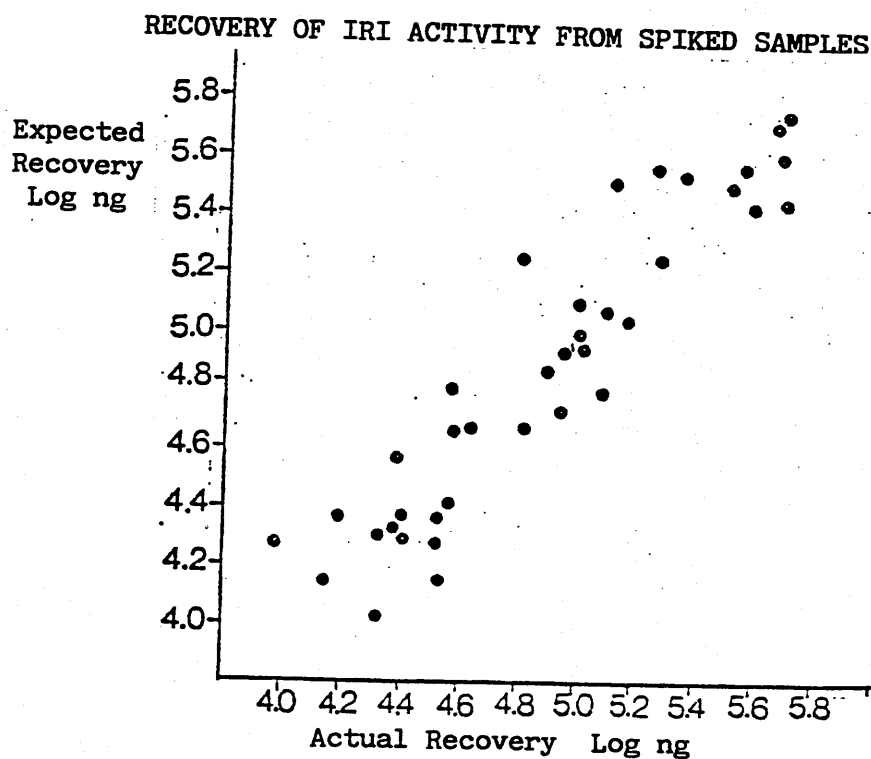


Figure 1

RECOVERY OF THE INSULIN CONTENT FROM
CHOPPED PANCREATIC TISSUE

Blade Advance Setting	Total Insulin Content of Chopped Tissue	Total Insulin Content of Supernatant	% Tissue Insulin Recovered
100 μ m	55,333	37,500	59.6
	23,591	7,760	75.25
	192,120	86,320	69.0
	47,325	20,665	69.61
	40,000	16,622	70.64
Mean \pm S.D.	71,674	33,773	67.97 \pm 5.7
250 μ m	81,330	39,000	67.59
	28,683	8,220	77.73
	146,320	40,182	78.46
	46,072	3,571	92.81
	51,580	14,186	78.43
Mean \pm S.D.	70,847	21,032	77.11 \pm 8.99
500 μ m	70,666	24,000	74.65
	34,908	5,560	86.26
	156,302	14,315	91.08
	68,175	4,052	94.39
	87,000	10,152	89.55
Mean \pm S.D.	83,410	11,616	87.78 \pm 7.59
750 μ m	112,000	20,500	84.53
	39,518	5,760	87.28
	160,840	15,820	91.02
	60,507	1,818	97.08
	85,000	8,956	90.47
Mean \pm S.D.	91,590	10,571	88.78 \pm 4.71
1 mm	120,000	25,000	82.76
	46,312	2,460	94.96
	146,940	11,350	92.83
	49,612	1,531	97.01
	76,000	14,364	84.1
Mean \pm S.D.	87,773	10,941	88.92 \pm 6.49
Control	139,666	7,200	95.05
	49,458	4,160	86.65
	128,523	8,946	93.49
	44,574	1,479	96.79
	62,500	12,149	83.73
Mean \pm S.D.	84,944	6,787	92.6 \pm 5.65

TABLE 8

Thus any variability in the amount of insulin measured (table 6) is due to differences in the insulin content of the pancreatic tissue evaluated and not to the methodology used .

MECHANICAL CHOPPING

In order to provide data on the dynamic aspects of insulin release from normal pancreatic tissue it was essential to chop the tissue into sufficiently small pieces to allow diffusion into and secretion of insulin out of the tissue fragments . It is clear that any tissue loss during this chopping procedure would to some extent invalidate these results.

I am aware of only one commercially available purpose built machine which has been used for chopping a variety of tissues . The result of using this McIlwain tissue chopper which makes parallel cuts transversing the tissue at pre-set intervals was investigated. It was found that if a rigid single edged blade was substituted for the recommended double edged razor blade and the blade force was set to maximum the human pancreas could be efficiently chopped into small fragments. The effect of using the McIlwain tissue chopper in regard to cellular damage and islet tissue loss was estimated on pre-weighed chopped tissue by measuring the insulin content of the chopped tissue and the insulin which was released during the chopping process i.e. the insulin content of the supernatant following washing of the chopped tissue. Several blade advance settings were used , the results of which are presented in table 8 .

The recovery of tissue insulin was calculated from the following formula .

$$\frac{\text{Insulin content of chopped tissue}}{\text{Insulin content of chopped tissue supernatant}} \text{ ng}$$

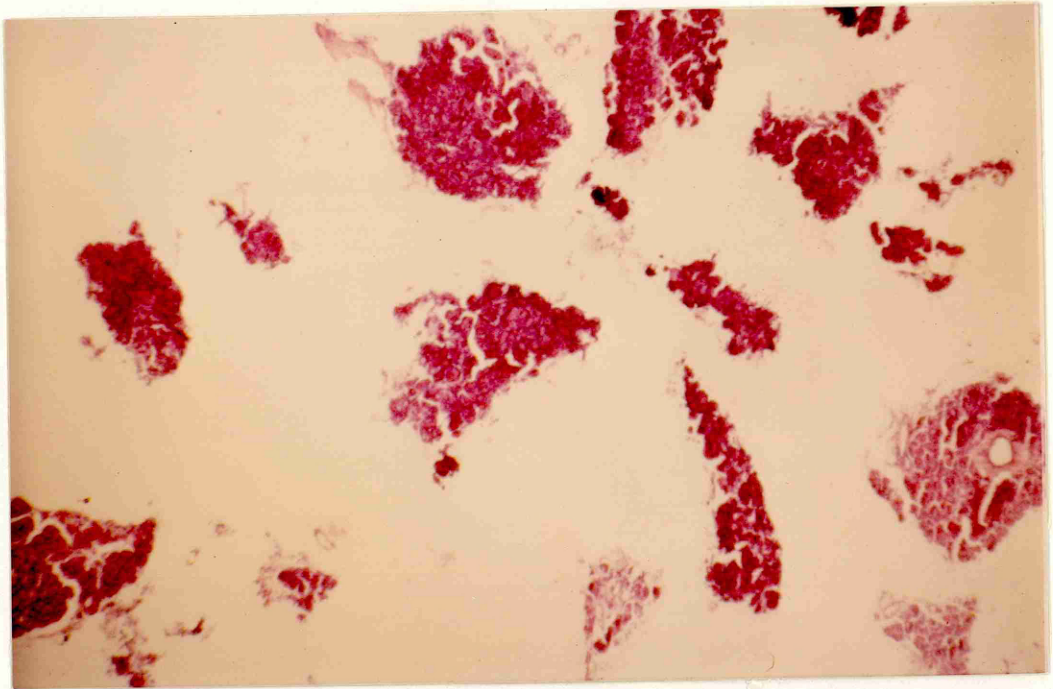
In addition histology examination of the chopped tissue was carried out (photographs 1 & 2 , page 92).

One interesting and perhaps not unexpected finding (table 8) was that there was a small but significant release of IRI from the unchopped control tissue following washing with cold balanced salt solution . Whether this was due to IRI release from cells prior to pancreatectomy or during the period of cold storage remains unclear .

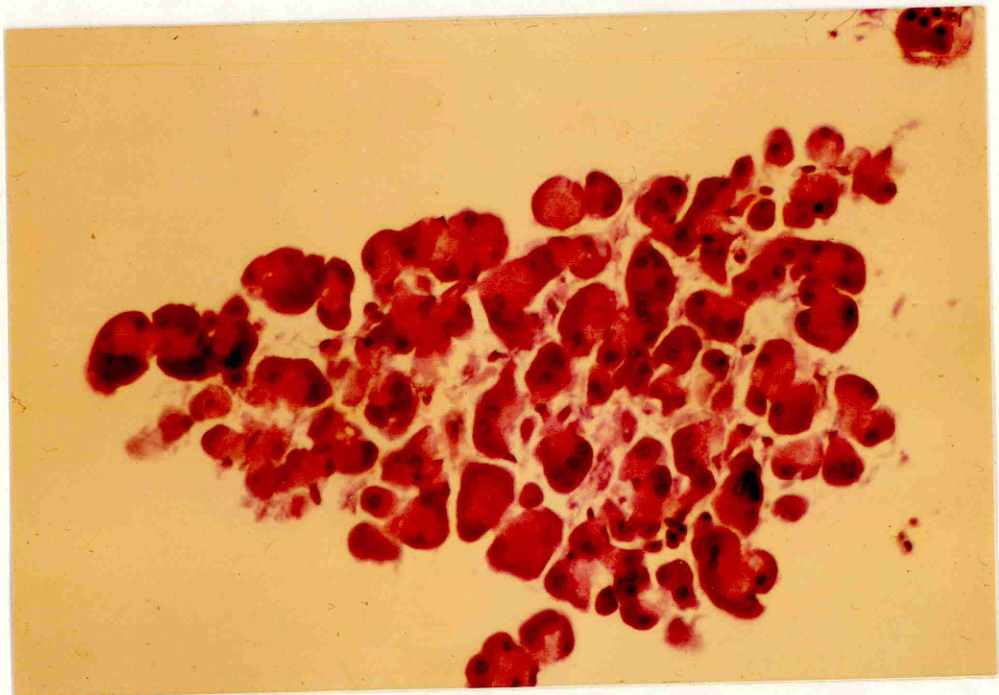
The results of the recovered tissue insulin content indicate that the human pancreas could be chopped into small pieces using a blade advance of between $500\mu\text{m}$ and 1mm with negligible loss of the insulin content from the tissue when compared to the unchopped control pancreas .The effect of mechanical chopping at lesser blade advance settings resulted in a 13% loss at $250\mu\text{m}$ which was increased to a 33% loss following chopping at $100\mu\text{m}$.

As can be seen from photographs 1 & 2 , there was no apparent morphological evidence of cellular destruction around the cut tissue edges when a blade advance setting between $500\mu\text{m}$ and 1mm was used . Thus the blade advance setting of $500\mu\text{m}$ was used to chop the pancreatic tissue throughout the following studies.

It remained to establish the insulin secretory capacity of this chopped tissue to establish the parameters needed against which to critically evaluate the islet function remaining following disaggregation.



Chopped Tissue
Haematoxylin and Eosin Magnification X 25
PHOTOGRAPH 1



Chopped Tissue
Haematoxylin and Eosin Magnification X 500
PHOTOGRAPH 2

PERIPERFUSION OF CHOPPED PANCREATIC FRAGMENTS

A known weight of randomly chosen tissue fragments from the chopped pancreas as above was gently introduced into the periperfusion system as described in Appendix III .

It was clear that there were considerable difficulties in the interpretation of the dynamic aspects of IRI release from the chopped pancreatic tissue and that certain aspects of this IRI release warranted explanation.

The results of those studies using media with a constant non-stimulatory (2.8 mMol) level of glucose are presented in Table 9 , page 94 (see also fig 4 , p.100). It can be seen that an initial extraordinary high IRI release during the early phase of periperfusion was followed by steadily declining rates of IRI release .

Although similar patterns of insulin release have been described following experimental periperfusion studies using other species (Downing 1981) , these have so far not been satisfactorily explained . Several suggestions have however been put forward.

1. That the apparent high insulin levels may be due to zymogen and enzyme release from the non-endocrine tissue leading to degradation of released endogenous insulin or the iodinated insulin used in the radioimmunoassay . Similarly these enzymes could lead to degradation of ,or loss of ,the labelled ligand from the insulin antibody used . These factors would of course lead to either false high or low estimates of IRI activity.

2. That the cell membrane integrity may be severely impaired and 'leakage' of insulin may occur and may possibly lead to deple-

PERIFUSION OF CHOPPED HUMAN TISSUE WITH CONSTANT LOW GLUCOSE (2.8 m MOL) GLUCOSE LEVELS
 IRI Release ng / min / g tissue

Time (mins)	Perifusion												Mean
	1	2	3	4	5	6	7	8	9	10	11	12	
5	500	500	118.0	84.9	500	183	-	362	412	500	128.6	315	328
10	288	157	138	68.5	500	68	111.2	-	306	188	27.2	217	188
15	220.5	276	96.7	41.1	500	58	50.5	122.5	318	83	14.9	264	159.3
20	164.7	178	96.2	40.1	383	44.4	-	142.5	255	57.7	13.3	248	147.5
25	172.1	176	60.3	40.3	185	29.4	33.7	-	211	222	7.5	118	114.1
30	154.6	196	23.4	53.3	190	29.4	60.5	74.3	180	100.5	10.1	96	97.7
35	153.5	124.5	19.4	67.9	225	27.4	74.6	59.8	118	72.7	8.0	94.2	86.6
40	159.1	129.5	38.5	73.9	130	28.7	68.3	47.8	122.1	58.8	10.1	80.4	78.2
45	155.7	108.7	59.0	111.0	118	25.3	27.9	45.2	117	72.9	8.9	72.1	75.0
50	142.3	107.1	49.3	91.2	153	25.3	52.4	30.1	100.5	106.9	13.9	58.4	79.7
55	144.5	82.9	54.6	82.1	132	23.6	96.7	35.1	72.6	-	16.5	74.3	74.1
60	137.2	72.2	41.9	72.1	221	27.4	47.3	31.9	60.9	78	10.7	60.0	71.7
65	146.8	80.5	-	75.0	225	17.8	72.2	18.9	45.8	-	6.9	54.3	74.3
70	97.5	65.6	38.3	64.6	310	23.3	-	17.3	45.2	119.7	8.5	56.0	76.9
75	119.9	64.3	36.4	45.7	196	17.4	22.8	21.2	41.2	118.0	9.1	52.8	62.1
80	141.2	47.3	43.5	43.1	346	16.1	77.4	16.4	53.8	99.9	13.9	118.3	84.7
85	145.6	58.9	57.2	28.0	248	15.4	-	22.5	-	100.8	18.1	56.4	75.1
90	127.7	70.1	36.5	23.4	212	12.6	45.3	21.2	35.2	120.1	16.5	39.5	63.3
95	172.5	81.3	45.4	25.1	258	9.2	28.7	26.2	29.5	132.5	12.8	64.2	73.8
100	110.9	76.3	60.9	23.8	203	8.5	35.3	18.3	26.8	72.7	10.7	57.3	65.0
105	98.0	56.4	-	25.4	183	9.6	-	28.5	30.5	80.3	10.7	44.2	56.7
110	114.2	61.4	26.8	15.3	151	9.2	29.6	45.0	31.0	39.5	10.7	43.5	48.1
115	96.4	70.1	-	15.3	170	13.0	24.2	29.5	30.0	106.9	14.4	44.0	55.8
120	105.3	33.6	22.5	10.8	192	12.6	17.6	24.6	34.2	53.2	12.8	52.3	47.6
125	94.7	36.5	-	12.3	108	9.9	20.3	16.8	27.9	76.9	14.9	54.0	42.9
130	107.6	36.5	16.3	9.6	116	9.6	17.5	22.0	-	171.0	16.0	49.3	51.9
135	97.5	21.9	12.9	9.4	142	6.2	29.4	17.2	23.9	-	16.0	58.0	39.5
140	117.6	44.8	12.8	11.1	124	6.8	-	17.5	-	72.7	11.1	62.0	48.1
145	72.6	43.9	17.2	-	98	6.5	17.8	25.3	37.3	85.5	14.9	59.3	43.4
150	-	48.5	13.5	-	111	9.6	-	-	-	-	10.1	60.0	42.2
155	58.3	36.2	-	-	-	10.6	-	21.3	26.2	96.2	8.5	46.3	37.9
160	62.1	38.4	-	-	121	10.3	-	-	42.4	80.3	6.9	51.1	51.5
165	55.3	20.5	-	-	-	7.9	-	-	37.5	61.5	10.7	-	38.9
170	40.8	11.8	-	-	-	4.4	-	18.6	32.4	79.2	9.1	-	30.9
175	40.6	-	-	-	-	5.8	-	-	33.5	84.0	2.1	-	33.2
180	-	-	-	-	60	6.2	-	-	58.3	-	6.5	-	32.7

TABLE 9

RECOVERY OF IODINATED INSULIN

Time (mins)	Experiment					Mean
	1	2	3	4	5	
5	53.7	66.9	74.0	52.4	55.3	60.5
10	71.8	79.0	78.9	59.4	62.3	70.3
15	75.0	85.4	84.5	77.4	66.0	77.7
20	85.4	88.4	87.2	75.3	88.0	84.9
25	89.4	85.5	89.2	68.3	90.3	84.5
30	85.1	89.5	90.9	91.8	100.7	91.6
35	80.1	87.6	87.2	85.1	92.3	86.5
40	87.8	83.1	91.5	93.9	88.7	89.0
45	79.3	89.3	94.6	91.9	87.7	88.6
50	89.6	82.5	88.4	79.3	87.0	85.5
55	73.5	84.9	86.3	81.3	93.3	83.9
60	76.6	89.8	91.6	77.3	88.0	84.7
65	89.1	84.3	82.9	77.2	78.3	82.4
70	67.0	90.5	75.4	84.3	101.0	83.7
75	68.3	89.4	82.8	83.5	89.0	82.6
80	84.5	88.4	80.0	93.3	99.7	89.2
85	94.1	86.6	91.2	74.1	82.0	85.6
90	62.3	84.2	76.1	86.9	96.7	81.2
95	85.9	90.8	90.6	78.2	89.0	86.9
100	86.2	87.0	93.3	91.7	90.7	89.8
105	72.7	88.4	93.3	81.6	86.7	84.4
110	79.0	84.7	84.3	84.6	90.3	84.6
115	81.8	89.1	88.4	78.3	86.2	84.8
120	81.1	89.6	88.7	85.9	88.0	86.7
Perfusate	81.1	86.9	87.5	82.2	89.0	85.3

TABLE 10

RECOVERY OF IODINATED INSULIN - ANTIBODY COMPLEX

Time (mins)	Experiment				Mean
	1	2	3	4	
5	91.9	84.4	92.0	87.8	89.0
10	91.6	85.2	92.0	98.9	91.9
15	93.1	98.4	91.1	84.6	91.8
20	86.5	90.4	109.8	94.1	95.2
25	102.1	89.8	102.9	98.1	98.2
30	100.1	115.8	89.8	96.2	100.4
35	96.8	104.9	96.9	105.1	101.7
40	103.6	105.1	98.9	114.7	105.5
45	100.7	97.3	103.1	102.2	100.8
50	107.5	102.9	116.0	107.1	108.5
55	112.8	111.0	86.7	91.2	100.4
60	111.8	112.9	93.6	100.3	104.6
65	94.5	116.9	94.0	90.6	99.0
70	109.2	107.2	94.2	86.7	99.5
75	114.3	87.8	97.6	86.7	96.6
80	94.2	90.6	118.1	95.1	99.5

TABLE 11

tion of insulin reserves.

3. A final reason may be that what one is observing is the progressive death of insulin containing cells during the period of perfusion.

It was possible to check the first assumption by two series of experiments. Iodinated insulin equivalent to $1,000 \mu\text{U ml}$ was added to the perfusate throughout the periperfusion. It has been shown that this can be absorbed by using a charcoal-dextran slurry (Herbert 1965) and more importantly that this absorbant is sensitive to damaged tracers (Hunter 1971). The recovery of iodinated insulin would indicate whether insulin degradation occurred. The results of these studies are shown in Table 10.

Similarly, factors which may have an adverse effect upon the insulin antibody used were investigated by incubating the collected perfusate fractions overnight at 4°C with a measured amount of the bound iodinated insulin-antibody complex. Because of the nature of the ligand antibody complex, the presence of free insulin in the perfusate would to some extent replace the labelled ligand from the antibody until equilibrium was reached. It was therefore necessary to remove the free insulin from the perfusate. This was carried out by incubating the perfusate fractions at 4°C overnight with an excess of insulin antibody. The antibody was removed by precipitation and centrifugation and the perfusate was then incubated overnight with the bound labelled ligand antibody complex. Once again a reduced recovery of the labelled ligand-antibody complex would suggest either damage to the antibody or the avidity of the bound ligand. The results of these studies are shown in Table 11.

It is clear from these figures that the recovery of iodinated insulin is reduced during the first 15 minutes of the periperfusion (Table 10). A similar effect was demonstrated from the initial 20 minute periperfusate on the recovery of the iodinated ligand-antibody complex (Table 11) .

The effect of this reduced recovery of the labelled ligand indicates that less ligand will be available to bind to the antibody complex and will therefore result in reduced counts in the precipitated antibody complex and falsely high estimates of the actual IRI activity during the first 15 minutes of the periperfusion. The high recovery of both iodinated and bound ligand during the remainder of the periperfusion also indicates that the measured IRI activity reflects an accurate estimate of the actual IRI activity .

It remains to be seen whether insulin release after the first 20 minutes of the periperfusion was the result of ' leakage ' as Sutherland (1981) suggests or whether active physiological secretion was occurring . No experimental model was set up which would determine whether 'leakage occurred ', although data to be presented later does not rule out its occurrence contributing to the initial high IRI release during the first 20 minutes of the glucose challenge.

One interesting finding was that the measured IRI release after the first 40 minutes of periperfusion could be inhibited to in excess of 60% by the addition of 10 μ g ml diazoxide (a substance known to inhibit insulin release in vivo). This finding argues against 'leakage' playing an important role in the

PERIPERFUSION USING CONSTANT LOW GLUCOSE LEVELS

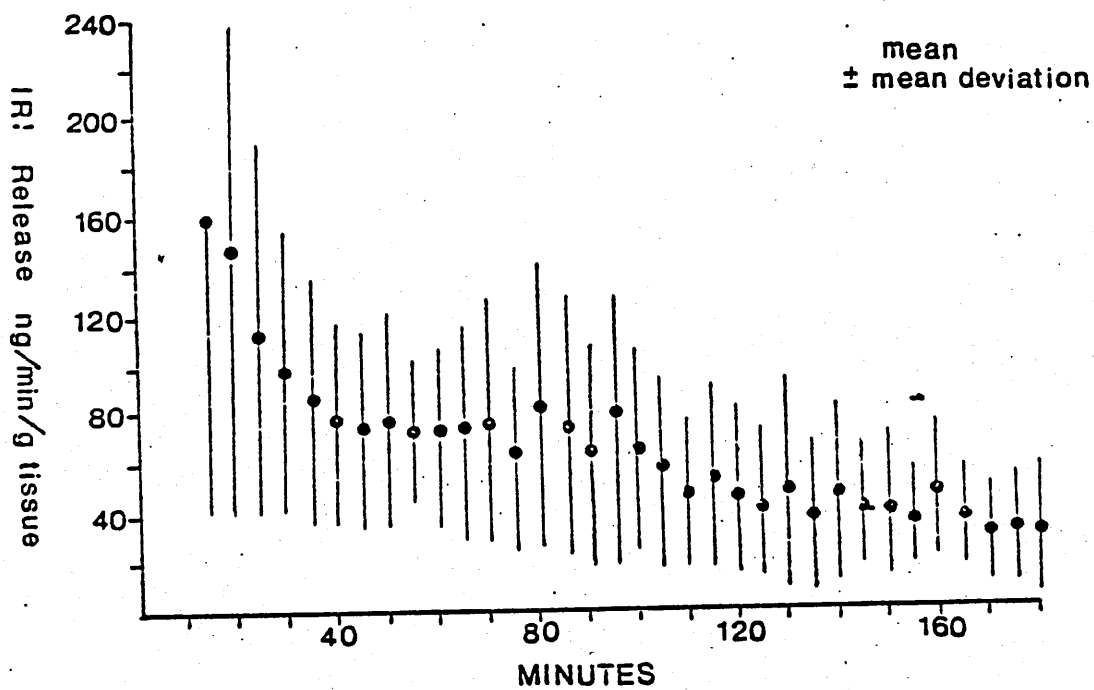


FIGURE 2

pattern of IRI release during the period of periperfusion .

The results of the periperfusion studies using non-stimulatory (2.8 mMol) glucose levels (shown in table 9 ,p.104) indicate that the individual values of insulin release exhibit a wide distribution ranging from 6.9 to 346 ng/ g tissue, similar to the wide range in tissue insulin content (table 6). This creates considerable problems in data analysis . The distribution has proved impossible to present in graph form without recourse to using logarithms or complex statistical procedures. In addition we cannot be sure that the few extreme values were not derived from the pancreas of donors who had abnormal pancreatic endocrine function .

For this reason and for the sake of clarity it appears sensible to use the arithmetic mean as our measure of central tendency and the mean deviation to demonstrate the range of values (Moroney 1951). It is realized that these rather simple statistical procedures are to some extent limited in their ability to deal with more advanced sampling theories such as are applied to normally distributed data but for the purpose of this study they would appear adequate .

The mean and mean deviation of the values obtained during the constant non-stimulatory glucose periperfusion (shown in Table 9) are presented in graph form in Figure 2 .

It is thus possible to make the assumption (although we cannot absolutely prove this) that the steady state of IRI release under constant non-stimulatory levels of glucose would indicate that normal physiological secretion was occurring and that islet cells were surviving during this period of

GLUCOSE CHALLENGE CARRIED OUT ON CHOPPED NON - DIGESTED TISSUE

IRI Release ng./min / g tissue

Time (mins)	Perfusion											Mean
	1	2	3	4	5	6	7	8	9	10	11	
2.8 mMol Glucose	185.5	500	-	500	-	142	-	500	-	116	34.0	282.5
	-	429	365	500	118.5	98	-	-	50.6	-	-	225.1
	74.8	283	51.6	-	-	60.5	-	-	20.6	52.5	30.4	213.7
	64.7	199	44.3	166.9	96.7	53.3	99.1	219.7	19.5	47.5	-	101.1
	63.3	329	48.7	-	-	46.3	-	-	28.3	43.2	32.6	84.5
	43.7	484	39.3	142.5	60.4	62.8	69.3	113.6	29.4	38.9	-	108.5
	38.3	283	21.5	129.3	-	74.9	-	-	20.9	42.7	36.1	87.1
	32.6	235	30.3	68.6	23.4	51.9	28.5	47.0	18.5	34.1	-	62.0
	33.9	290	38.2	60.0	-	54.8	-	-	18.5	35.8	40.3	71.4
	29.4	180	24.0	60.0	19.3	62.8	24.9	82.5	23.5	30.9	-	54.2
	26.5	133	28.5	52.8	41.9	70.7	22.0	-	16.5	23.4	12.8	48.4
	26.5	145	30.0	42.6	31.7	52.0	-	59.2	18.3	31.2	-	51.3
	27.8	133	-	43.7	38.4	26.6	18.2	71.6	8.3	28.6	10.6	40.7
	25.3	173	25.2	-	36.4	29.3	21.3	80.9	21.5	24.4	16.4	51.9
16.8 mMol Glucose	113.2	337	41.7	78.8	79.3	108	32.7	223	138.0	54.6	20.3	111.4
	38.4	375	49.5	60.0	43.5	112.4	50.5	193	73.5	48.3	23.8	97.1
	33.6	422	32.1	121.7	57.2	83.0	24.9	238	33.9	54.6	26.4	96.0
	30.6	302	21.3	-	36.5	63.2	-	212	61.8	28.5	-	93.8
	-	216	25.5	77.2	45.1	62.8	32.0	246	214.0	19.3	21.5	95.9
	21.4	265	27.3	78.8	60.9	83.0	52.6	204	195.0	22.6	23.2	93.9
	-	231	41.7	-	50.5	62.2	-	204	115.2	24.6	19.3	93.5
	21.4	202	54.6	199.6	33.8	53.5	55.6	196	38.5	20.4	-	87.5
	34.5	244	49.5	126.8	60.4	72.0	53.6	217	28.7	19.7	35.7	90.6
	-	272	37.4	-	30.8	85.4	-	156	40.3	19.5	32.4	87.7
	21.4	320	32.7	126.8	-	79.2	42.0	200	32.8	17.3	43.8	86.9
	19.7	252	32.5	138.9	26.8	58.3	50.4	-	63.8	21.3	-	106.0
	18.6	314	32.5	-	-	90.4	-	181	88.7	29.3	36.4	98.8
	20.6	357	43.2	127.6	22.5	102.2	55.2	155	-	36.1	-	102.1
	37.8	264	48.0	99.4	-	68.3	53.4	181	61.0	33.0	30.5	87.6
2.8 mMol Glucose	-	-	35.1	-	-	-	-	177.6	-	27.3	12.9	63.2
	-	133	44.7	-	-	-	-	-	-	29.6	-	69.1
	-	-	30.3	-	-	-	-	75.6	-	-	17.5	41.1
	-	112	20.7	-	-	-	-	-	-	21.1	-	46.2
	-	170	32.5	-	-	-	-	68.2	-	20.0	12.2	60.6
	-	-	6.5	-	-	-	-	75.4	-	27.5	16.5	68.9

TABLE 12

periperfusion . In terms of confirmation that normal islet cell function was occurring it was also necessary to show that this tissue responded to elevated levels of glucose with an increase in IRI release.

For this reason the glucose challenge was carried out in the following manner .

After a 30 minute period of periperfusion in which $10\mu\text{g/ml}$ diazoxide was added to the perfusate to inhibit insulin release, a non-stimulatory glucose level of perfusate (as described in the methodology in Appendix III) was introduced . This was changed to a 16.8 mMol glucose level of perfusate after 30 minutes and once again 2.8 mMol glucose was re-substituted after a further 30 minutes. The perfusate was collected at two minute intervals.

The results of these studies shown in Table 12 , and the mean IRI release and mean deviation are presented in Figure 3 , p. 99 .

Once again a high initial rate of IRI release was observed using 2.8 mMol/l glucose . This declined after the first 20 minutes of the glucose challenge to a relatively steady rate of IRI release ranging from 8.3 to 145 ng/min/g tissue with a mean of 49ng/ min/ g tissue .

In all experiments the rate of IRI release could be shown to increase in response to an increased glucose concentration of 16.8 mMol. The mean IRI release over the 30 minute periperfusion using 16.8 mMol glucose concentration was found to increase to 95 ng / g / tissue and more importantly from the point of view of

GLUCOSE CHALLENGE OF CHOPPED HUMAN TISSUE

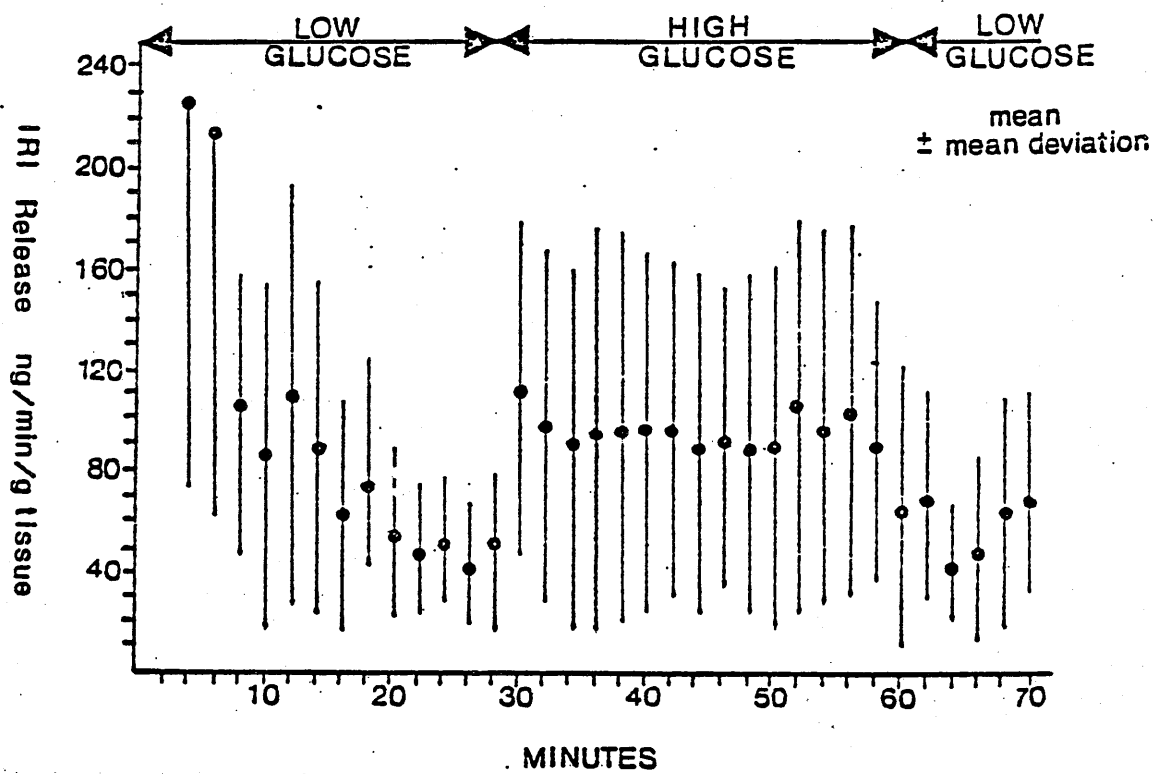


FIGURE 3

quantifying this potentiated IRI increase this was shown to be sustained over the 30 minute period.

The final 10 minute periperfusion using 2.8 mMol glucose resulted in a decrease in IRI release to a mean of 54.2 ng / min /g tissue.

It was clear that the pattern of IRI release did not (or as is more likely the periperfusion design was insufficiently sensitive to) demonstrate the primary and secondary peaks of IRI release reported when rodent isolated islet preparations (Lacy 1972) and whole organs (Landgraf 1971) have been perfused . It is likely that mixing within the periperfusion chamber and the time taken for diffusion of glucose into and IRI release out of the tissue may have contributed to masking this reported cyclic

GLUCOSE CHALLENGE ON STORED TISSUE

		Period of storage of whole gland in ice					
		6 Hours n = 4		8 Hours n = 4		24 Hours n = 2	
Time (mins)		Mean	Mean Deviation	Mean	Mean Deviation	Mean	Mean Deviation
Low Glucose	2	349.0	132.0	525	-	-	-
	6	163.0	67.0	88.3	20.0	-	-
	10	66.5	27.0	41.5	21.0	75.8	42.0
	14	25.1	5.0	26.5	14.0	135.2	75.0
	18	23.5	4.0	22.3	11.0	44.0	11.0
	22	19.0	13.0	18.0	6.6	17.7	11.7
	26	24.2	11.0	16.9	6.0	4.5	3.6
High Glucose	30	47.2	13.0	13.6	6.7	3.6	0
	34	38.8	7.0	12.4	3.7	1.2	1.2
	38	44.3	10.3	14.2	5.3	0	0
	42	38.9	11.0	13.9	5.0	0	0
	46	35.3	7.3	14.1	4.0	0	0
	50	36.7	13.4	11.3	4.0	0	0
	54	31.3	6.0	11.8	3.6	0	0
	58	34.9	8.0	12.2	4.6	0	0
	62	20.4	6.0	9.6	0	0	0

TABLE 13

pattern of IRI release.

For the purpose of this study, the analysis of primary and secondary peaks of IRI release was not considered essential and the steady state of IRI release under both basal and stimulatory levels of glucose was more amenable to quantitative estimates of islet function .

The constant rate of IRI release under basal glucose and the increased response to elevated levels of glucose provide two important measures of endocrine function . These of course also provide the parameters which allow us to critically evaluate the effect of the proposed techniques of human pancreatic disaggregation both in terms of the quantity and quality of remaining islet function.

4.2 TISSUE DISAGGREGATION OF THE HUMAN PANCREAS

Effects of storage;

As previously mentioned (Appendix I) it was impossible to control the length of storage in ice of the pancreas . Nevertheless , it was possible to attempt to evaluate the effect of prolonged ice storage on endocrine tissue survival using the periperfusion technique .

Cold storage of the whole gland in ice led to the progressive loss in the ability of the tissue to produce a sustained IRI release during a glucose challenge (Table 13).

Periperfusion of pancreatic tissue fragments following 6 hrs cold storage showed a decreased level of IRI release both in response to low and stimulatory levels of glucose .The tissue

GLUCOSE CHALLENGE OF STORED CHOPPED TISSUE

	Time (mins)	24 HOUR STORAGE at 4 C (n = 3)		CRYOPRESERVATION (n = 7)	
		Mean	Mean Deviation	Mean	Mean Deviation
28 m Mol/l Glucose	2	817.0	354.0	-	-
	6	289.0	59.0	-	-
	10	137.0	27.0	75.9	27.6
	14	75.0	45.6	45.5	17.6
	18	40.6	25.6	26.7	7.5
	22	23.1	9.3	21.5	7.2
	26	19.9	9.0	19.5	5.9
168 m Mol/l Glucose	30	43.7	23.3	27.0	10.5
	34	40.0	18.3	26.5	14.6
	38	46.2	20.3	20.9	13.9
	42	39.3	14.6	23.8	15.3
	46	39.0	15.3	20.0	12.5
	50	40.0	22.0	19.9	11.8
	54	30.1	7.3	16.6	10.3
2.8	58	29.8	7.0	21.2	14.0
	62	19.6	-	16.1	10.6
	66	-	-	12.8	4.5

TABLE 14

did however respond to stimulatory glucose concentrations with increased IRI release indicating that a percentage of islet tissue survived for this period of time . Following 8 hours storage in ice a further reduction in IRI release was recorded, and no increase in IRI release could be shown in response to increased glucose levels suggesting that damage to the normal physiological response of the endocrine tissue had occurred. In the two studies in which the pancreatic tissue was stored in ice for 24 hours no IRI release could be demonstrated after 20 minutes periperfusion suggesting that the tissue had failed to survive .

In contrast in the four studies in which the pancreas was chopped into small fragments within three hours of pancreatectomy and stored in tissue culture media at 4°C a degree of the IRI secreting capacity of the pancreatic fragments could be maintained following storage for up to 24 hours (Table 14).

An alternative method of preservation was attempted by the storage of pancreatic tissue fragments cryopreserved following the procedures outlined in Appendix IV . A markedly reduced level of IRI release was however measured following thawing 24 hours later (Table 14) .

These combined results are presented in Figure 4 .

It is clear from these studies that none of the strategies used were effective in totally preserving endocrine function and the loss of endocrine function was rapid and progressive . If processing of the pancreas has to be delayed for any reason then the most favourable conditions in terms of the preservation of

GLUCOSE CHALLENGE ON STORED CHOPPED PANCREAS

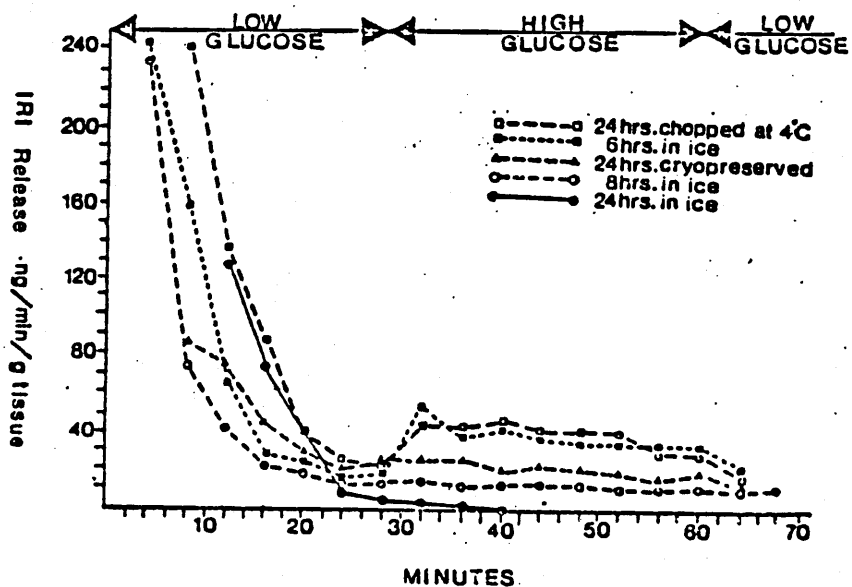


FIGURE 4

endocrine function would appear to be as chopped fragments stored in culture media at 4°C.

Tissue Distention:

In the following studies attempts were made to initially disrupt the tissue prior to chopping by injection of cold (4°C) digestion solution (Appendix III).

Ductal distension was performed by injecting 50 ml of the distention solution into the pancreatic duct . Histologic examination (Photograph 3) indicated that there was no apparent damage to the pancreatic islets . The most striking finding was the grossly dilated ductal system and it would appear that distention was confined to these vessels as no distention or

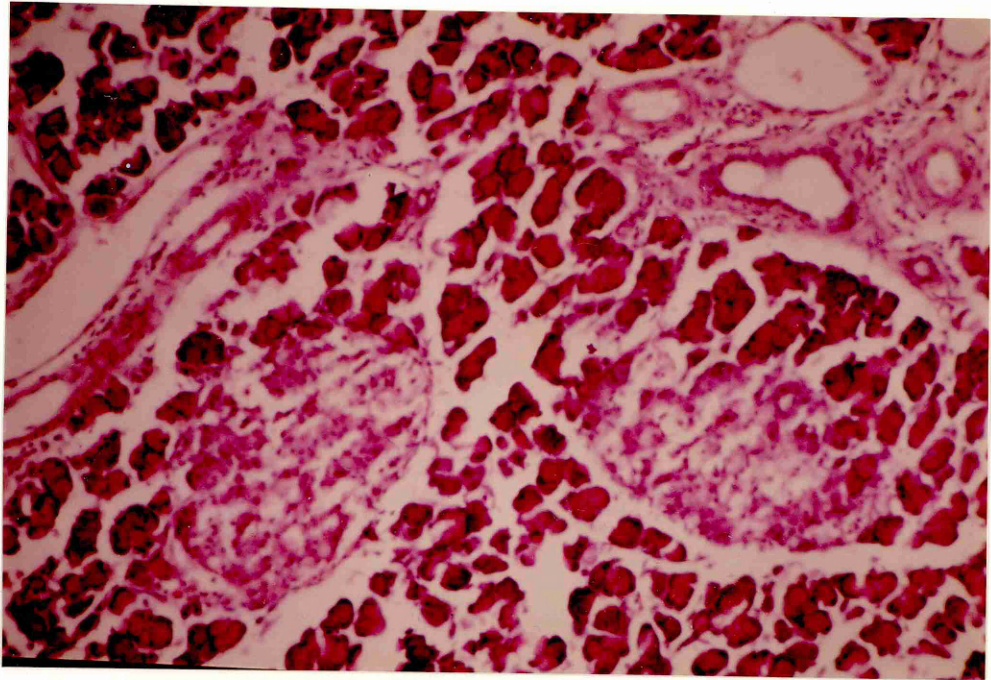
disruption of the tubular or acinar endpieces were observed. Ductal distention would therefore seem completely ineffective in producing the desired tissue disruption.

The superior mesenteric artery was cannulated and all major tributaries of the pancreatic veins were clamped. The gland was distended by injecting 50 ml of the cold distention solution. Histological examination of the tissue was carried out, from which it was clear that intra-arterial distention resulted in the total disintegration of islet tissue (Photograph 4).

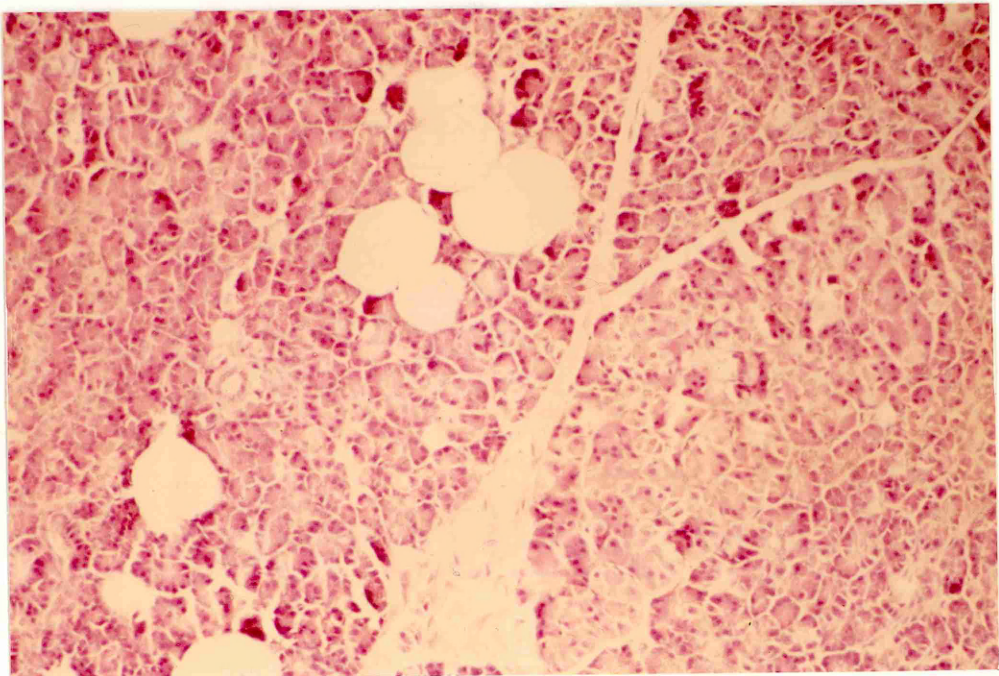
Venous distention of the pancreas was carried out by injecting 50 ml of distention solution into the superior mesenteric vein after all other major blood vessels had been clamped. Histological examination indicates that excellent disruption of the pancreatic tissue stroma both around the acini and ductal tissue can be achieved. In addition, it can be seen that there was complete disruption of the tissue stroma surrounding the islets (Photographs 5 & 6).

These findings would indicate that venous distention provides considerable advantages in its ability to initially disrupt the tissue stroma and was therefore performed on all pancreatic tissue prior to digestion.

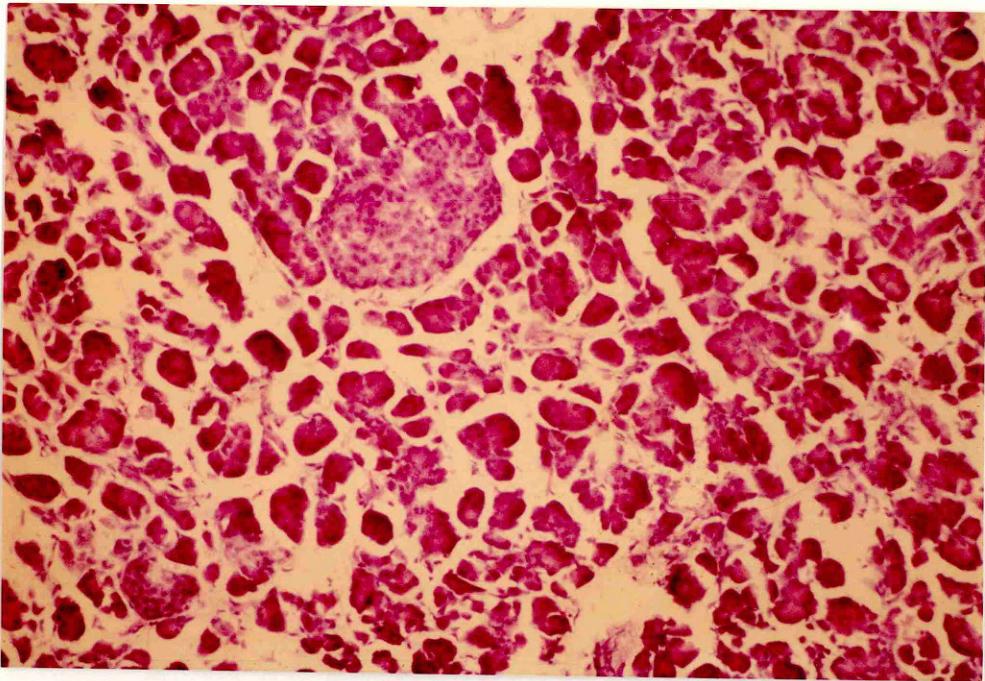
Out of interest the reason why venous distention would appear to be so effective was further studied by adding indian ink to the culture media used for distending the gland. Histological evidence of a 20 μ m unstained section of the pancreas (Photograph 7) demonstrates the extensive venous network within the pancreas and the 6 μ m stained section (Photograph 8) shows that the distention solution can be seen



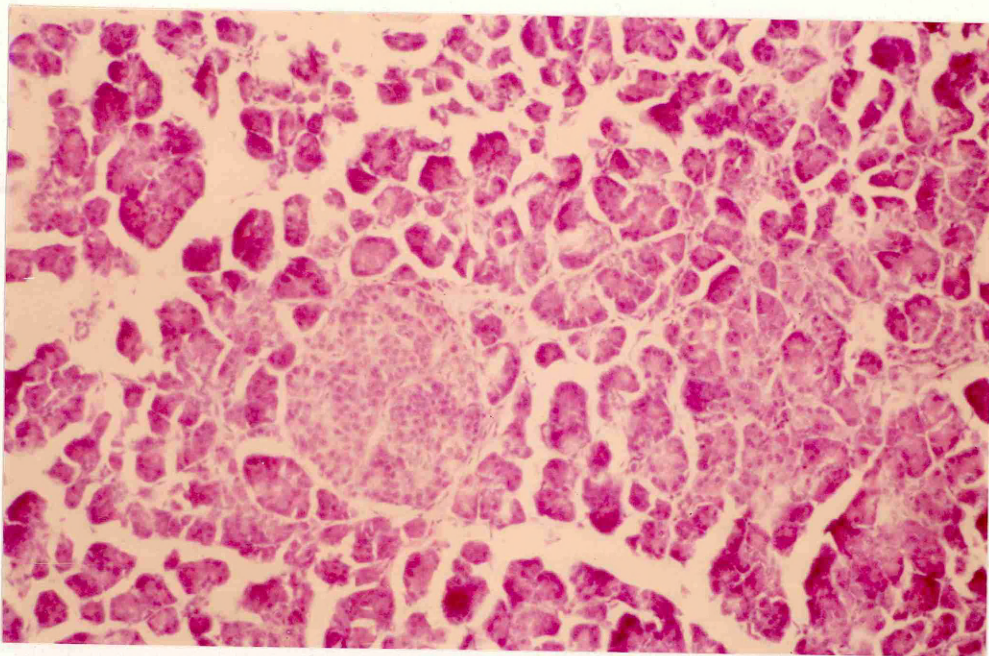
Arterial Distention of the Human Pancreas
Haematoxylin & Eosin Magnification X 20
PHOTOGRAPH 3



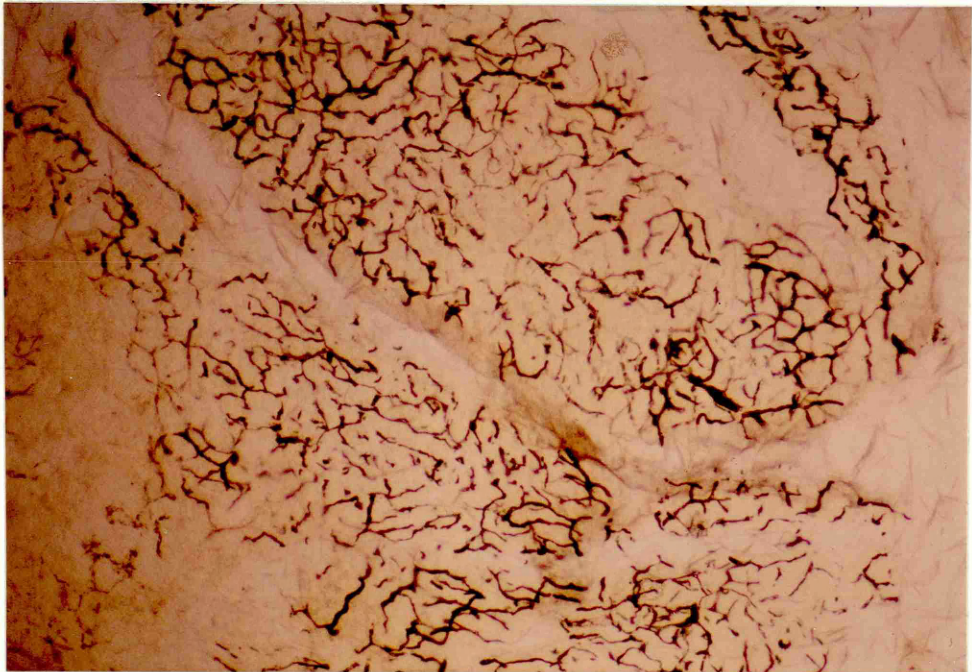
Ductal Distention of the Human Pancreas
Haematoxylin & eosin Magnification X 20
PHOTOGRAPH 4



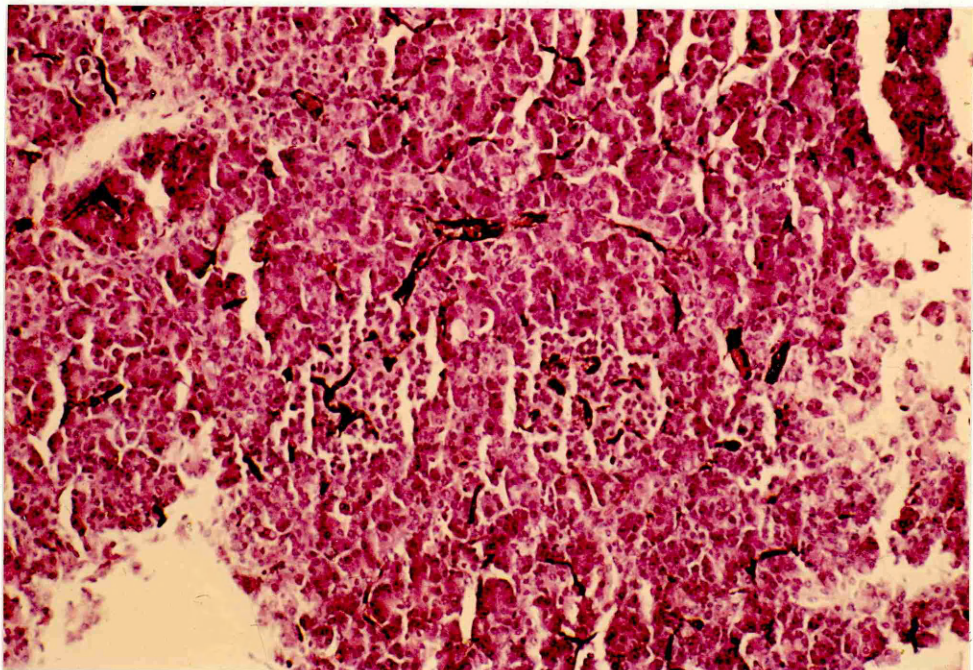
Intravenous Distention of the Human Pancreas
 Haematoxylin & Eosin Magnification X 100
 PHOTOGRAPH 5



Intravenous Distention of the Human Pancreas
 Haematoxylin & Eosin Magnification X 250
 PHOTOGRAPH 6



Intravenous Injection of Indian Ink
Unstained 20 m section Magnification X 5
PHOTOGRAPH 7



Intravenous Injection of Indian Ink
Haematoxylin & Eosin Magnification X 40
PHOTOGRAPH 8

reaching what on morphological grounds would appear to be the efferent arterioles of the islets . Thus if sphincters separating the endocrine-exocrine components exist in the human pancreas then they must be located within the pancreatic islets.

Following venous distention , the pancreas was carefully dissected free of fat , major blood vessels , ductal tissue and the pancreatic capsule .

The pancreas was then sliced into 3 to 4 mm slices and chopped using the McIlwain tissue chopper at $500\mu\text{m}$. This has been described in detail in part 4.1 .

These procedures took on average slightly in excess of 30 minutes during which time the tissue was maintained at as near to 4°C as possible.

Enzymatic Digestion

Since our primary aim in using enzymatic digestion was to achieve islet isolation while preserving islet function , we needed to answer the question of which enzymatic components were effective in tissue disaggregation and the relative effect of these components on islet cell survival.

The detailed methods and results employed to fractionate and purify these enzymes are presented in Appendix V .

Various fractions of the eluate following ultragel fractionation were used to digest chopped tissue for 40 minutes at 37°C . The degree of tissue disaggregation was estimated by filtering the tissue remaining following digestion through a nylon mesh with a $250\mu\text{m}$ pore size .

The average percentage of the total tissue which would

FILTERING OF DIGESTED TISSUE

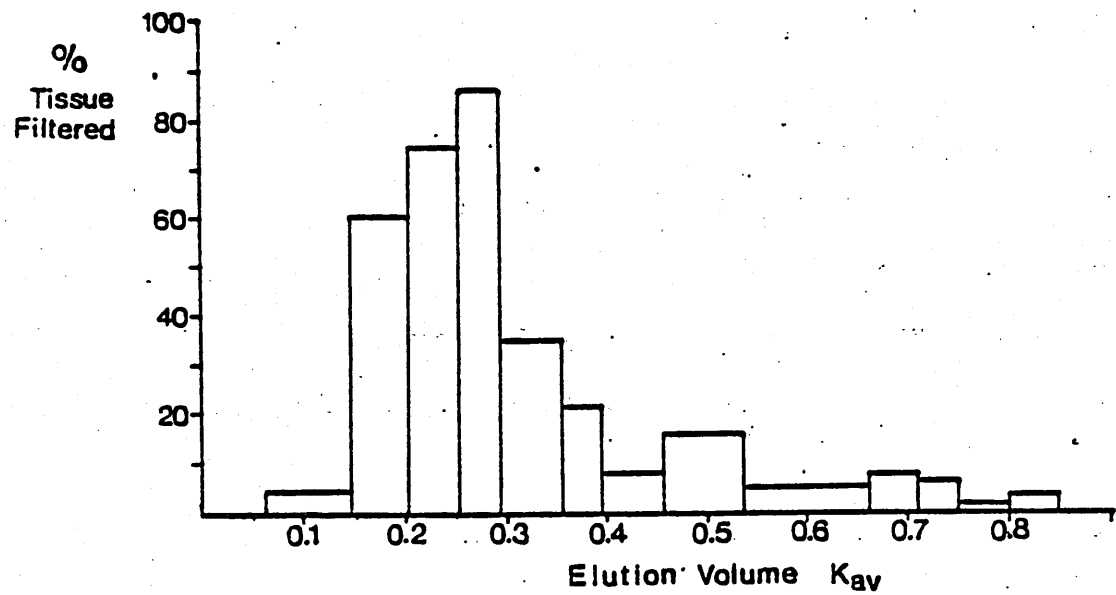


FIGURE 5

COLUMN FRACTIONATION OF CRUDE COLLAGENASE

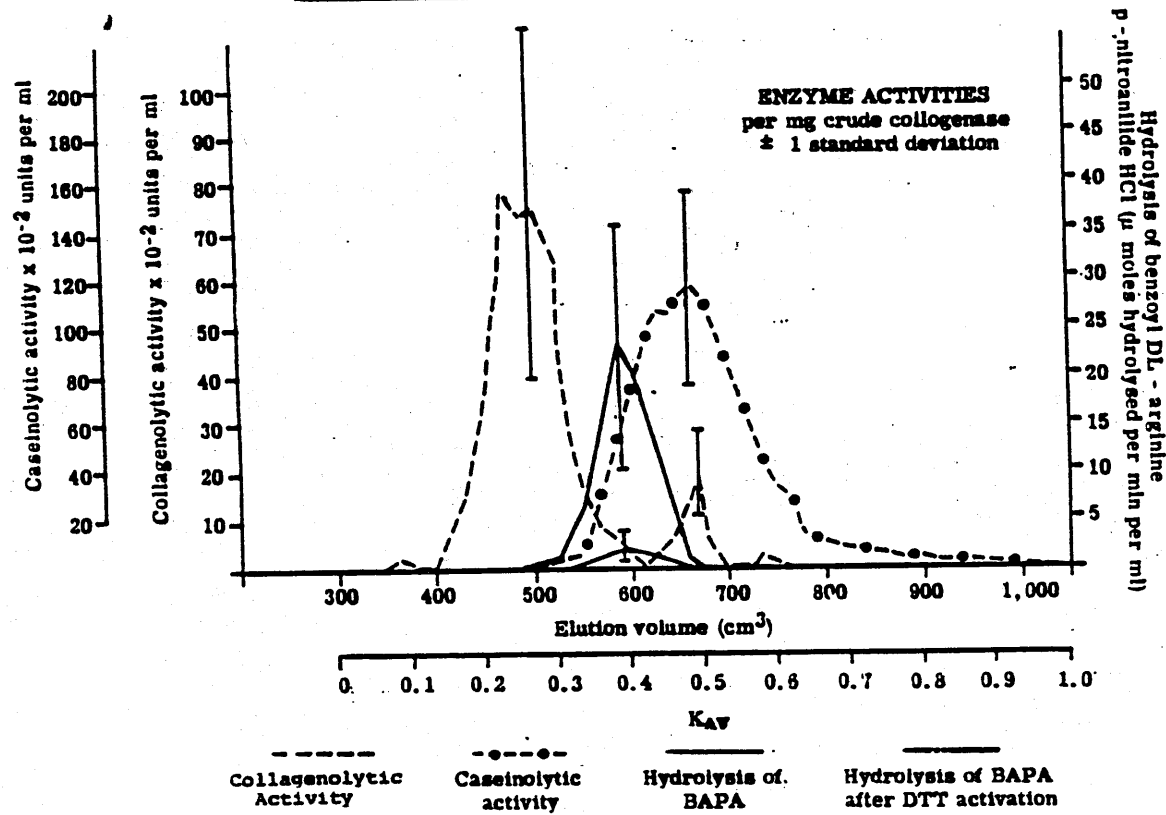


FIGURE 6

pass through this size mesh is plotted against the K_{AV} of the column eluate (Figure 5).

It was clear that only two areas of the column eluate were effective in achieving tissue disaggregation. The first which eluted between K_{AV} 0.13 and 0.35 exhibited only collagenolytic activity . The second although less effective peak eluted between K_{AV} 0.40 and 0.53 and contained collagenolytic, tryptic and non- specific protease activity.

When the histogram of filtered tissue (Figure 5) is compared with the enzymatic elution profile following gel filtration (Figure 6) it is clear that the degree of tissue disaggregation closely follows the presence and concentration of collagenolytic activity.

Purified preparations of the amidase-esterase , clostripain and the three isolated non-specific proteases (isolated following the procedures outlined in Appendix III) had no significant effect in achieving tissue disaggregation and islet isolation even when used at high concentrations.

The effect of these enzymes on individual cell release were studied further by estimating from aliquots of the digestion solution the number and viability (using dye exclusion) of those cells which were released during this period of digestion.

As a further indication of the effect of these individual enzymes histological evaluation of the non-disaggregated tissue was carried out following 40 minutes digestion .

The cell counts and viability measures of the cells released following 40 minutes digestion are presented in Table 15

CELL COUNTS ON ALIQUOTS OF DIGESTED TISSUE

K_{AV}	Cells $\times 10^4$				Viability			
	Exp..1	Exp.2	Exp.3	Exp.4	Exp.1	Exp.2	Exp.3	Exp.4
0.1-0.15	4	-	-	1	95	-	-	95
0.15-0.2	32	12	-	3	95	95	-	95
0.21-0.25	46	21	42	15	95	80	95	95
0.26-0.3	57	16	29	22	95	95	95	95
0.31-0.35	18	8	8	21	95	60	90	95
0.36-0.4	7	10	11	10	95	60	90	90
0.41-0.45	4	5	14	7	95	80	50	95
0.46-0.5	5	15	4	28	95	80	90	95
0.51-0.55	4	14	10	10	95	80	90	95
0.56-0.6	14	25	35	16	20	90	90	95
0.61-0.65	12	20	16	16	20	80	50	50
0.66-0.7	5	20	10	11	20	60	50	80
0.71-0.75	5	-	9	11	70	-	50	95

TABLE 15

CELL COUNTS AND VIABILITY OF CELLS
RELEASED DURING THE DIGESTION

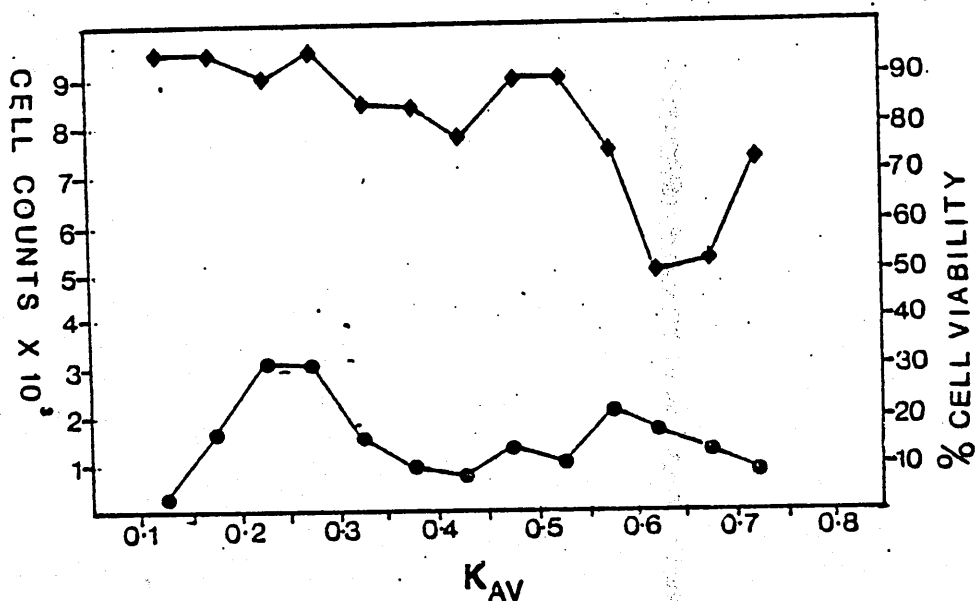


FIGURE 7

and the mean cell recovery and viability is presented in Figure 7.

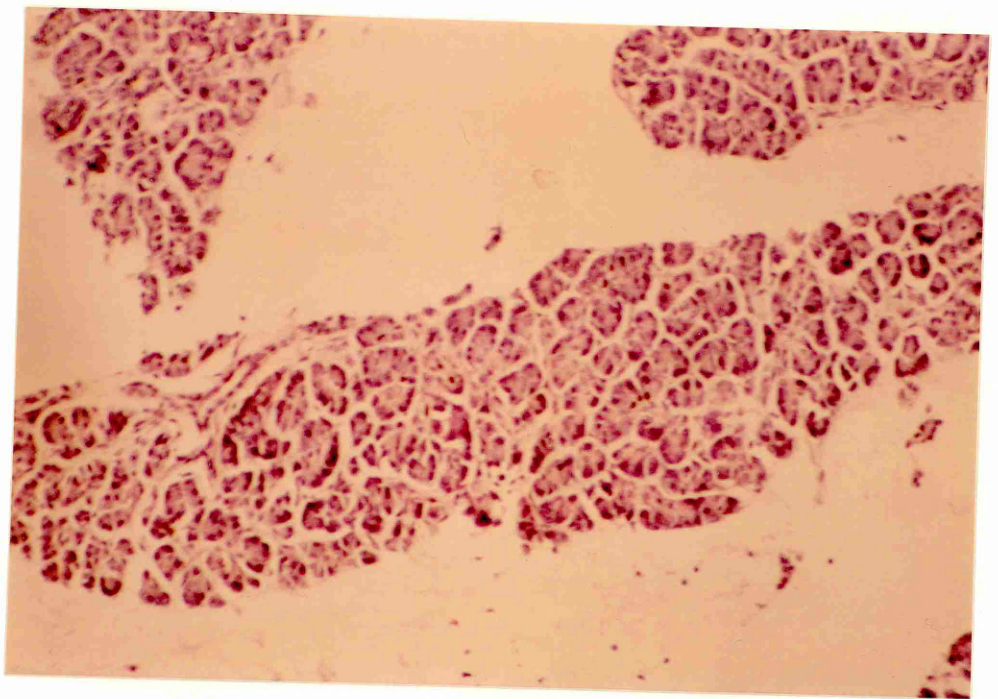
It is clear that each of the column fractions was capable of releasing individual cells and acini from the tissue during the digestion period. The greatest release of cells occurred when fractions eluting between K_{AV} 0.2 and 0.3 were used. Although considerable numbers of cells were released using column fractions eluting between K_{AV} 0.55 and 0.65 it would appear from the dye exclusion results that considerable cell death had occurred.

Histological evidence suggests that the digestion solution was capable of maintaining normal tissue morphology throughout the period of digestion (Photograph 9). The use of 100 units per ml of a purified collagenase preparation (Photograph 10) suggests that this enzyme was capable of achieving islet isolation and that no gross signs of tissue damage were observed. In contrast histological evidence of tissue fragments partially digested with purified preparations of clotripain, or the three non-specific proteases (Photographs 11,12,13 and 14) all show evidence of massive areas of non-discriminatory tissue damage.

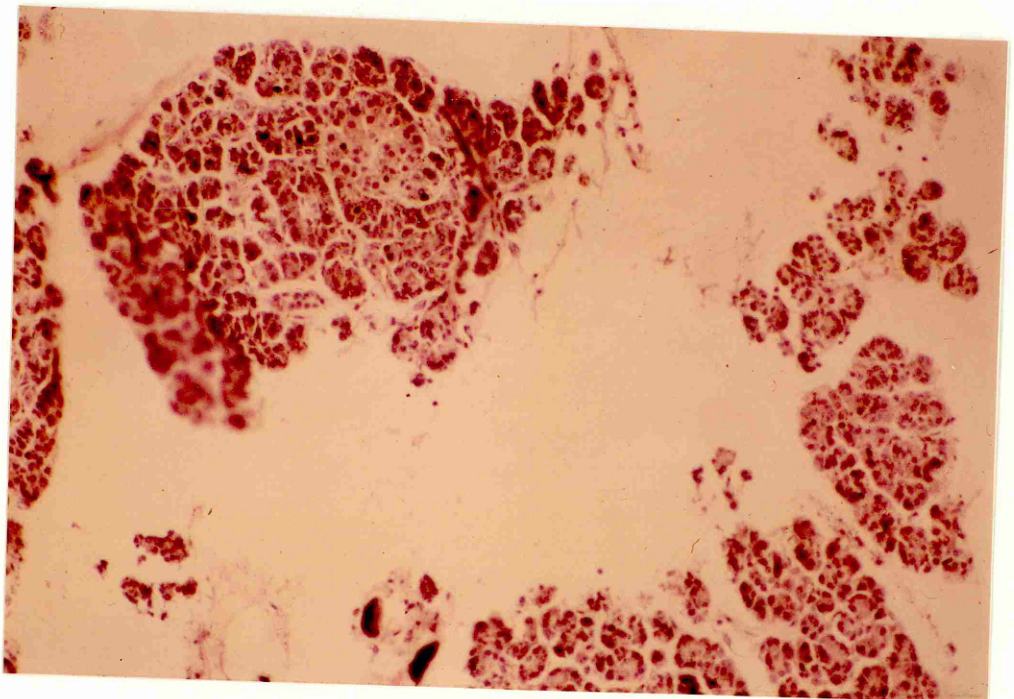
Taken together these findings indicated that the use of a purified clostridial collagenase preparation was sufficient to effect tissue disaggregation and provided considerable advantages over the use of the crude collagenase enzyme complex.

The digestion procedure which evolved using this pure enzyme preparation was as follows.

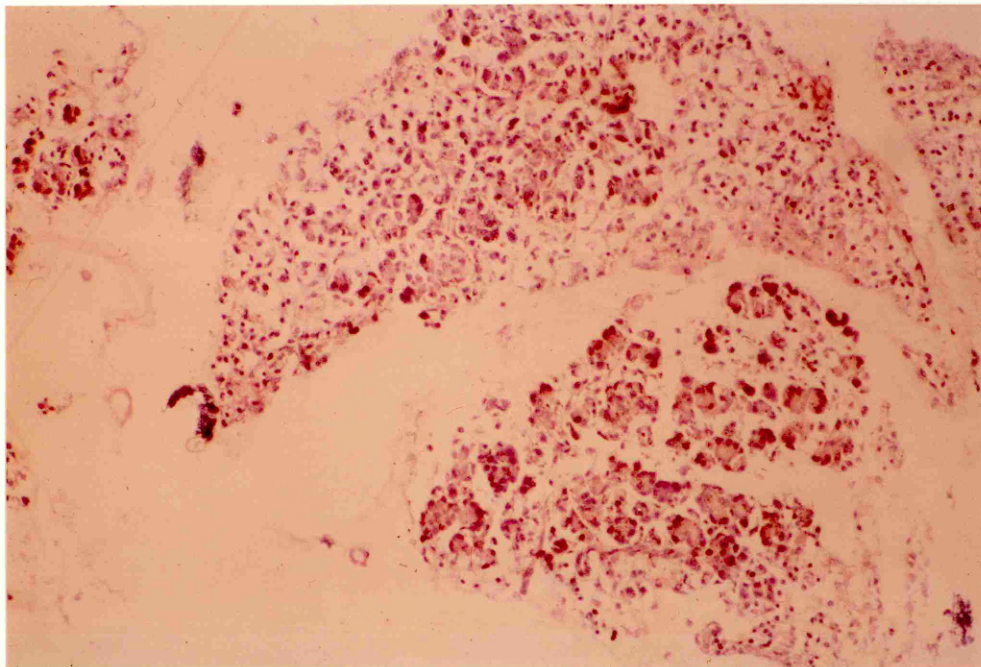
The chopped tissue was washed with two changes of the



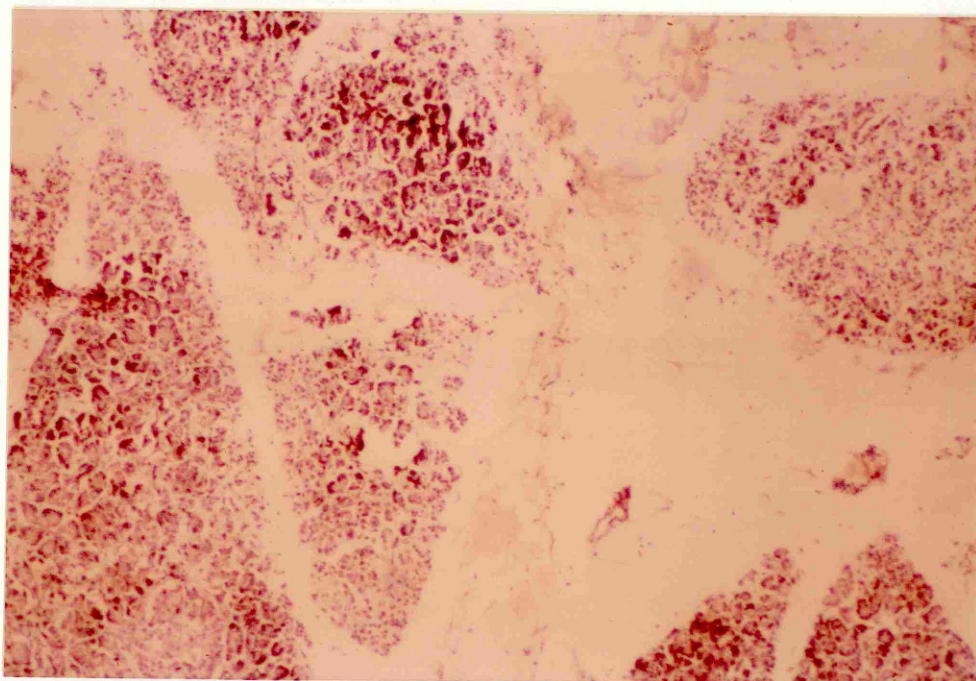
Human pancreas - following 40 minutes incubation
in the digestion soln
Haematoxylin & Eosin Magnification X 40
PHOTOGRAPH 9



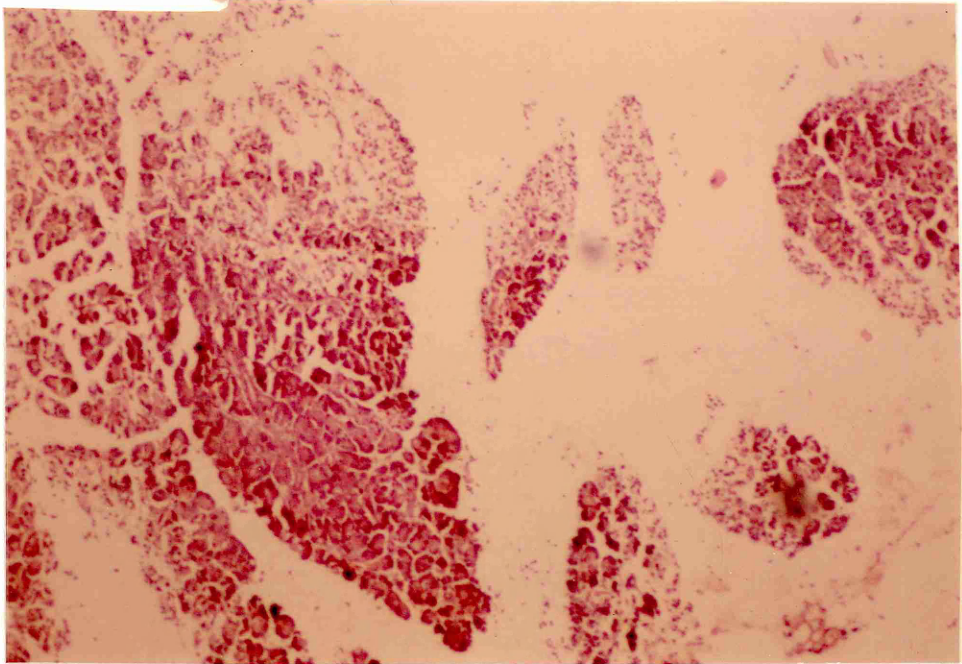
Human pancreas - following 40 minutes incubation
with pure collagenase
Haematoxylin & Eosin Magnification X 40
PHOTOGRAPH 10



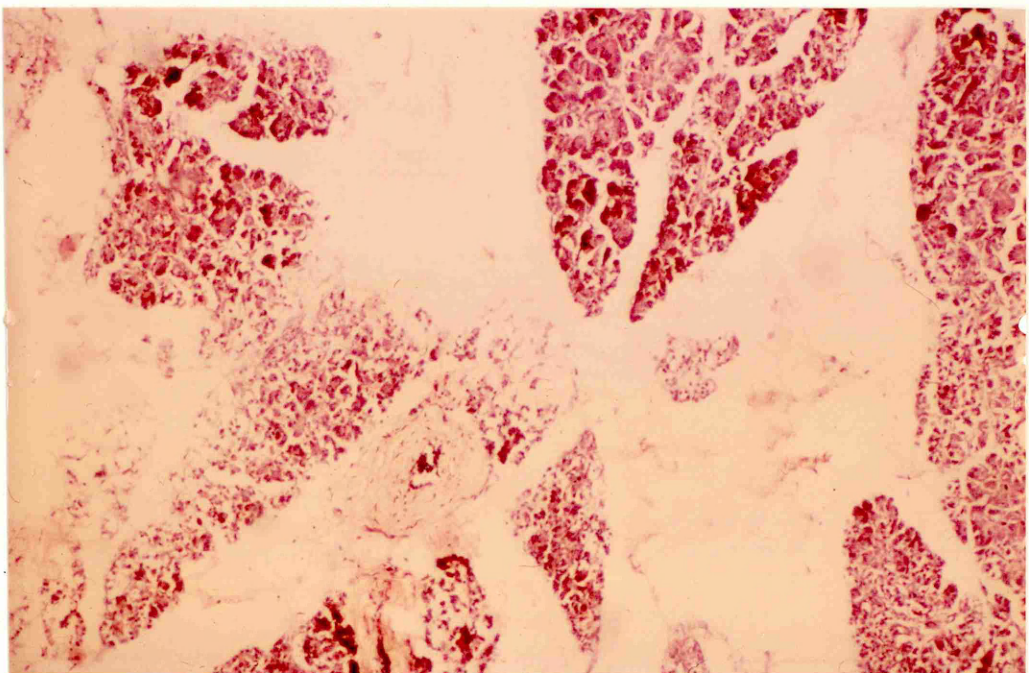
Human pancreas - following 40 minutes incubation
with clostripain
Haematoxylin & Eosin Magnification X 25
PHOTOGRAPH 11



Human pancreas - following 40 minutes incubation
with non-specific protease ($P_i = 3.7$)
Haematoxylin & Eosin Magnification X 25
PHOTOGRAPH 12



Human pancreas - following 40 minutes incubation
with non-specific protease ($Pi = 4.8$)
Haematoxylin & Eosin Magnification X 25
PHOTOGRAPH 13



Human pancreas - following 40 minutes incubation
with non-specific protease ($Pi = 7.5$)
Haematoxylin & Eosin Magnification X 25
PHOTOGRAPH 14

digestion solution (Appendix III). It was then incubated in ten times the tissue volume with digestion solution in a conical flask containing between 400 and 600 Units per ml purified collagenase. During digestion the tissue was maintained at 37° C and constantly mixed using a rotary shaker oscillating at 60 cycles per second.

The digestion was carried out in two stages . The first stage was limited to 30 minutes at which time the tissue could be seen to be breaking up. At the end of this period the tissue was allowed to settle for two minutes and the suspended tissue fragments were removed by decanting the digestion solution .The tissue fragments were harvested and washed using RPMI 1640 to remove the digestion solution by centrifugation at 500 g for 5 minute periods .

A second digestion was carried out using a fresh enzyme solution . The second digestion was allowed to continue until it was considered that complete tissue disaggregation had occurred i.e. only a minimal amount of tissue fragments was not in suspension . The time taken to achieve this degree of disaggregation varied considerably . In some cases disaggregation was complete after 15 minutes of the second digestion however occasionally pancreatic tissue proved resistant to complete dispersal even after 30 minutes in which case recourse was made to gentle pipetting to disaggregate the remaining tissue using a wide bore movette pipette (Flow Laboratories).This disaggregated tissue was again harvested and washed with RPMI 1640 using centrifugation at 500g for 5 minutes .

One point relating to the digestion process is of note. In

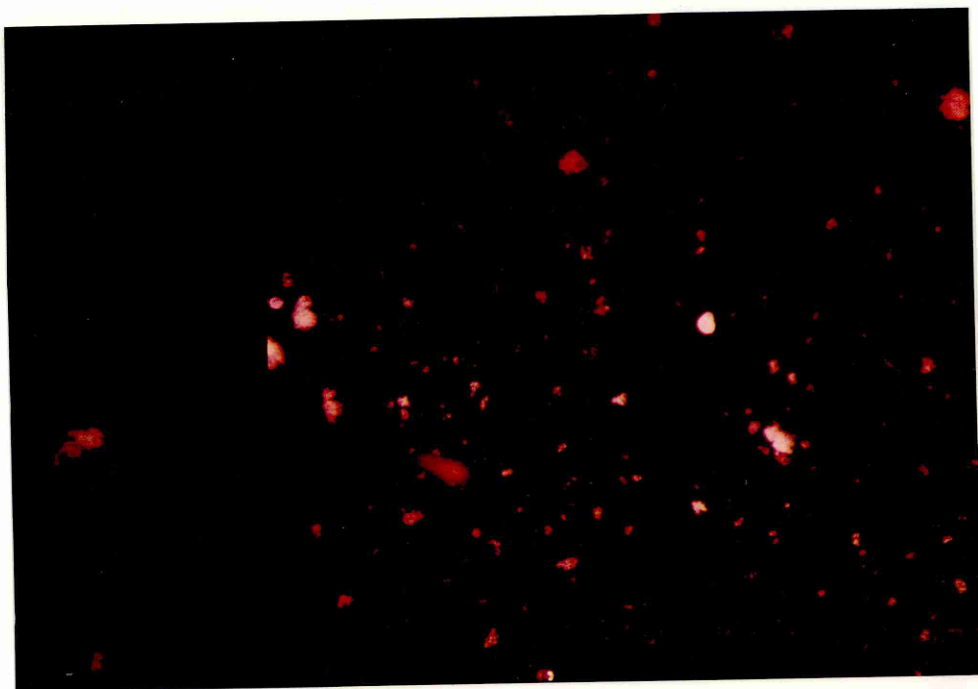
early studies there was a propensity for the disaggregating pancreatic tissue to form clumps due to the formation of a mucoid substance during the digestion period. A similar type of mucoid formation is a well recognized consequence of tryptic digestion (Waymouth 1974) and is thought to be caused by spindle extrusion from cells damaged during the digestion. It has been reported that the formation of this mucoid substance can be prevented by adding DNAase during the digestion. It was found that soya bean trypsin inhibitor had a similar effect but that α -1-antitrypsin (a more general protease inhibitor extracted from human serum) was notably more effective and was added during both stages of digestion at a concentration of 100 μ g / ml . We are aware of the reported inhibitory effect of α -1-antitrypsin on collagenolytic activity but in control studies no prolongation of the time needed to achieve tissue disaggregation was noted .

Examination of the unfixed tissue using incidental illumination of the disaggregate against a black background (Photograph 15) showed what would appear to be islets .

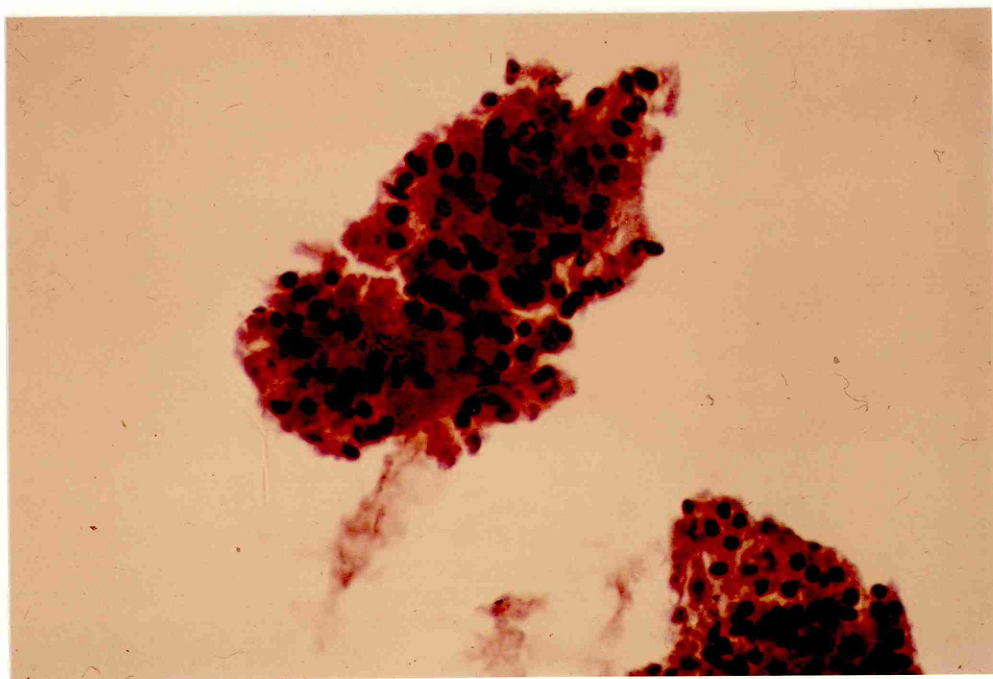
Histology of these presumed islets confirmed the endocrine type nature of the cells within these structures (Photograph 16).

For completeness Photographs 17 and 18 are presented to show the histological appearance of the dispersed pancreatic tissue at the end of enzymatic disaggregation and harvesting .

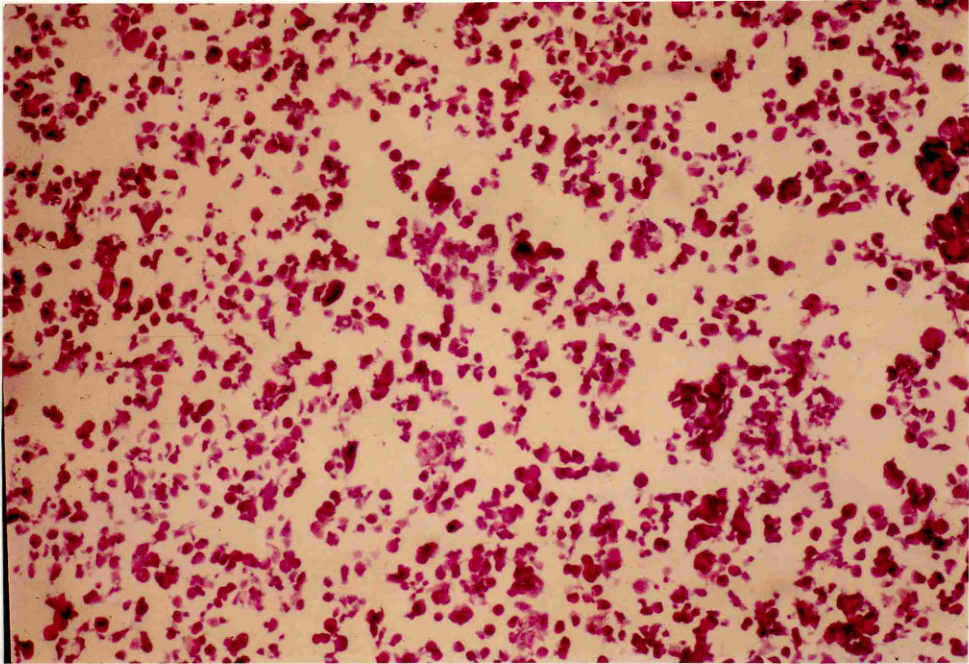
It now remains to evaluate the recovery and viability of this disaggregated tissue using the criteria developed on normal human adult pancreatic tissue developed in Chapter 4 .1 .



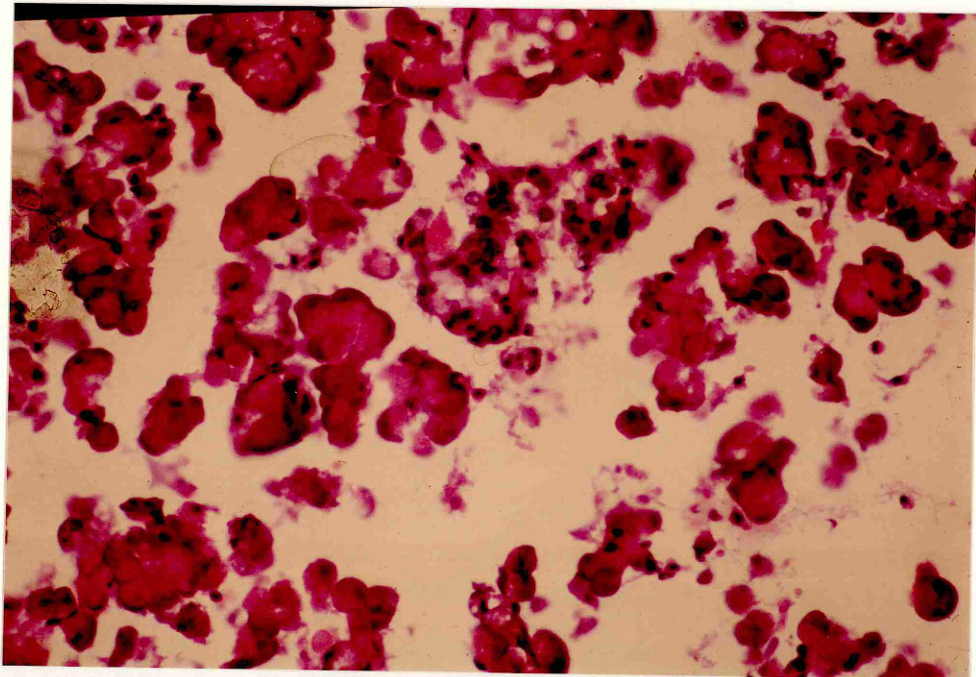
Pancreatic disaggregate - incidental light
 Magnification X 10
 PHOTOGRAPH 15



Isolated Islets
 Haemotoxylin & Eosin Magnification X 250
 PHOTOGRAPH 16



Disaggregated human pancreas
Haematoxylin & Eosin Magnification X 20
PHOTOGRAPH 17



Disaggregated human pancreas
Haematoxylin & Eosin Magnification X 200
PHOTOGRAPH 18

4.3 RECOVERY OF HUMAN PANCREATIC TISSUE AND ENDOCRINE FUNCTION

In twelve experiments weighed random aliquots of the chopped pancreatic tissue were subjected to enzymatic disaggregation exactly as described above .

Complete disaggregation occurred in each case and the harvested tissue was washed , weighed and the tissue insulin content was estimated as described (Appendix II).

These results are presented below in Table 16.

RECOVERY OF PANCREATIC TISSUE AND INSULIN CONTENT

Experiment No.	Original wt. of tissue gm.	Wt. of Disaggregate gm.	% Tissue Recovery	Insulin content of disaggregate ng/g/tissue
1	5.156	3.916	75.9%	204,602
2	4.927	3.947	79.9%	30,000
3	1.731	1.123	64.9%	90,000
4	1.436	0.995	69.4%	107,388
5	1.848	1.442	76.9%	76,000
6	1.662	0.988	59.4%	98,604
7	2.814	1.557	55.3%	184,000
8	2.499	1.660	66.5%	31,000
9	2.637	1.854	70.0%	640,000
10	7.635	5.865	76.8%	18,500
11	4.085	3.212	78.6%	26,480
12	4.162	2.448	59.8%	135,530
Mean	3.383	2.416	69.4%	136,842

TABLE 16

Thus on average we recovered 69.4% of the tissue by weight with a range of 55.3 % to 79.9 % recovery over the 12 samples used . The mean recovery of the insulin content of this disaggregated tissue was found to average 136,842 ng/g/tissue which was not significantly different $T = 23$ using the Whitney Mann rank order correlation coefficient) from the value obtained

GLUCOSE CHALLENGES CARRIED OUT ON ENZYMATICALLY DISAGGREGATED TISSUE

IRI Release ng / min / g tissue

Time (mins)	Perfusion												Mean
	1	2	3	4	5	6	7	8	9	10	11	12	
2.8 mMol Glucose	2	31.6	185.5	500	-	-	142.0	52.5	500	-	116	34.0	229.0
	4	27.4	-	429	500	118.4	47.7	-	-	-	-	-	225.5
	6	16.0	74.8	283	51.6	-	60.5	38.1	-	20.6	52.5	30.4	69.7
	8	18.5	64.7	199	44.3	166.9	53.5	35.5	217.7	19.5	47.5	-	76.7
	10	22.5	63.3	329	48.7	-	46.3	39.9	-	28.3	43.2	32.6	72.6
	12	13.8	43.7	484	39.3	142.5	60.4	34.3	113.6	29.4	38.9	-	108.5
	14	12.3	-	283	21.5	129.3	-	28.0	-	20.9	42.7	36.1	87.1
	16	14.3	32.6	235	30.3	68.6	23.4	23.0	47.0	18.5	34.1	-	62.0
	18	18.5	33.9	290	38.2	60.0	-	23.4	-	18.5	35.8	40.3	61.3
	20	18.5	-	180	24.0	60.0	19.4	31.8	62.5	23.5	30.9	-	50.6
	22	13.7	26.5	133	28.5	52.8	41.9	27.1	-	16.5	23.4	12.8	40.6
	24	11.8	-	145	30.3	42.6	31.7	24.5	59.2	18.3	31.2	-	44.7
16.8 mMol Glucose	26	9.6	27.8	133	-	43.7	38.3	23.5	71.6	8.3	28.6	10.6	38.4
	28	-	-	173	25.2	-	36.4	54.6	88.9	21.5	24.4	16.4	52.2
	30	47.9	113.2	337	41.7	78.8	79.3	108.0	223	138	54.2	20.3	118.1
	32	33.2	38.4	375	49.5	60.0	43.5	112.4	193	73.5	48.3	23.8	92.1
	34	21.3	33.6	422	32.1	121.7	57.6	83.0	238	33.9	54.6	26.4	96.0
	36	14.2	30.6	302	21.3	-	31.5	63.2	212	61.8	28.5	-	79.6
	38	16.1	-	216	25.5	77.2	45.1	62.8	246	21.4	19.3	21.5	87.6
	40	11.8	21.4	265	27.3	78.8	60.9	83.0	204	19.6	22.6	23.2	85.0
	42	14.8	-	231	41.7	-	50.4	62.2	204	11.5	24.6	19.3	78.6
	44	9.1	21.4	202	54.6	199.6	33.8	53.5	196	38.5	20.4	-	77.3
	46	7.0	34.5	244	49.5	126.8	60.4	72.0	217	28.7	19.7	35.7	76.3
	48	9.0	-	272	37.4	-	30.8	85.4	156	40.3	19.5	32.4	70.8
2.8 mMol Glucose	50	10.4	21.4	320	32.5	126.8	-	79.3	200	32.8	17.3	43.8	82.4
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	64	8.7	-	-	30.3	-	-	33.8	75.6	-	-	17.5	33.2
	66	11.5	-	112	20.7	-	-	27.9	-	-	21.2	-	38.7
	68	11.0	-	170	32.5	-	-	22.8	68.2	-	20.0	12.2	48.8
	70	-	-	-	20.5	-	-	23.1	70.1	-	27.5	16.5	31.6

TABLE 17

with fresh normal tissue .

It was then necessary to evaluate the remaining endocrine function of this pancreatic disaggregate using the periperfusion design presented in chapter 4.1 .

In a further 12 experiments randomly sampled aliquots of chopped pancreatic tissue averaging 2 g in weight were digested as above . The recovered tissue was washed , weighed and then gently introduced into the periperfusion system . Glucose challenges were carried out exactly as described for the chopped tissue fragments (chapter 4.1). The results of these periperfusions are presented in Table 17 and Figure 8 .

GLUCOSE CHALLENGE ON ENZYMATICALLY DISAGGREGATED PANCREAS

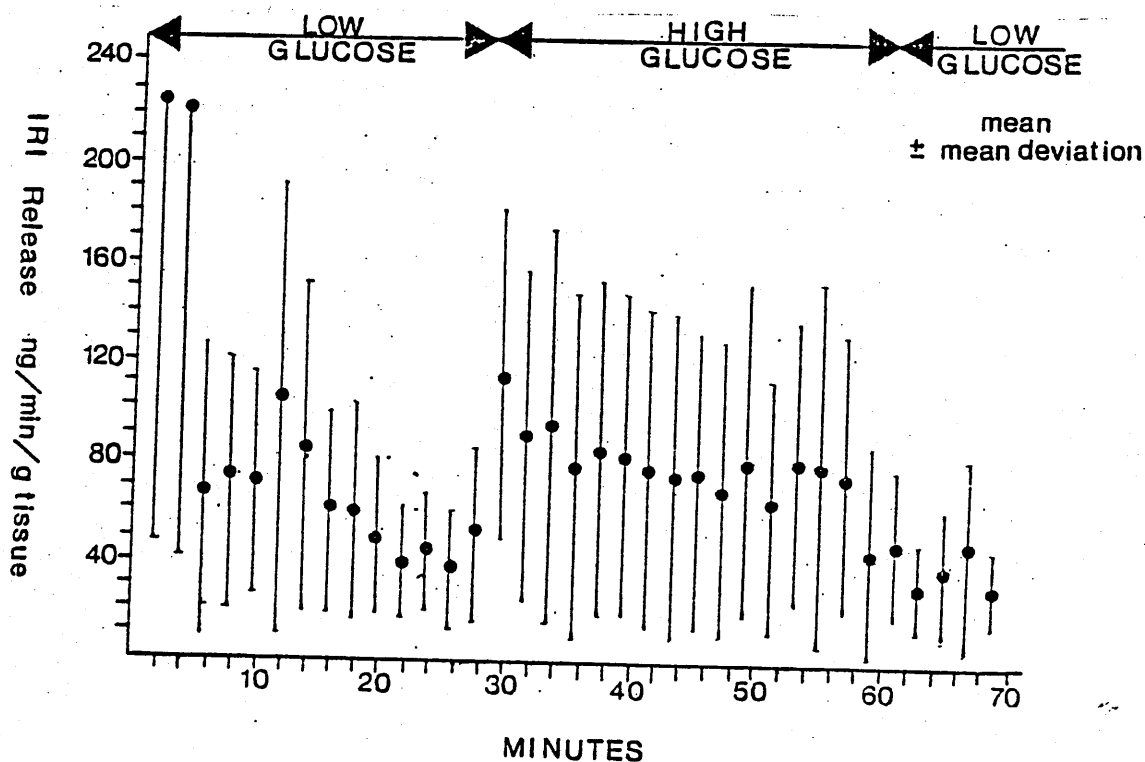


FIGURE 8

These results clearly indicate that a sustained IRI release occurred during the periperfusion and that this was potentiated in response to elevated glucose levels. In terms of the rate of IRI release this was comparable to chopped but non-digested tissue.

The conclusion must be that on average 69% of the IRI content of the pancreas was recovered following disaggregation and that following the criteria developed during periperfusion studies the endocrine function would appear to have been preserved.

SUMMARY and CONCLUSIONS ,

In the studies on normal human pancreatic tissue the most striking finding was the extreme variation in the IRI content between individual whole pancreas (table 6).

This finding is not confined to this study . The reported mean and standard deviation of the insulin content of the normal human pancreas ($140,000 \pm 120,000$, Sutherland 1975) and the mean and standard error of the rat pancreas ($84,000 \pm 24,000$, Lazarow 1973) would suggest similar between sample variations. The findings of Maitland (1980) of a similar between sample variation in age matched foetal pancreas may even indicate that a genetic predisposition towards this varied distribution exists.

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been shown (Appendix IV) to be accurate to within $\pm 3\%$ to which should be added possible errors in dilution.. The constancy in tissue and amylase content of the whole pancreas (table 6) suggests that errors in dilution did not introduce significant errors in the estimation of tissue IRI levels .

The recovery experiment carried out using adjacent portions of the pancreas (Table 7) indicates that the methodology used to extract IRI content was reasonably accurate with an overall recovery of 96.7% . The individual recoveries however varied considerably . It therefore follows that the sometimes large discrepancies in individual IRI levels in Table 7 could only have arisen from within tissue variations in the IRI content.

In view of this large between and within tissue variation it is perhaps not surprising that the periperfusion studies should also exhibit a wide variation in non-stimulatory IRI release .

It was established using constant non-stimulatory levels of glucose that criteria needed to be established in order to provide a reproducible and reliable estimate of the released IRI activity .

It was important to establish criteria in deciding which parameters would be indicative of 'viable' islet tissue . Perhaps the only valid criteria which could be argued to apply was that the tissue had retained its normal secretory potential in response to its main secretagogue glucose. This means that as in vivo the tissue being periperfused should show a constant IRI release in response to non stimulatory glucose and a potentiated and sustained increase in response to stimulatory levels of

glucose .

As can be seen from the constant non-stimulatory glucose periperfusion of chopped normal human pancreas (Table 9) there was (as in reported periperfusions using rodent pancreas) an initial surge in IRI release over the first 30 to 40 minutes . Iodinated insulin degradation and damage to or loss of avidity within the antibody-label complex (Tables 10 & 11) during the first 30 to 40 minutes suggested that measurements during this period were inaccurate and should be disregarded.

In 9 out of 11 pancreas periperfused with non-stimulatory levels of glucose (Table 9) the IRI release remained reasonably constant between 40 and 100 minutes . Glucose challenges carried out during this period using alternate low and high glucose concentrations (Table 12) showed that in 9 out of 12 experiments the dynamic aspects of IRI release paralleled the known in vivo response of islet tissue and therefore satisfied the criteria for viability above.

It remains unknown why in 75 % of periperfusions the tissue failed to satisfy the criteria for viability . It may be that these tissue preparations may have differed in their endogenous zymogen or enzyme components or that even the relatively short ischaemic times may have damaged the tissue .

What is clear from Figure 4 is that diminution of IRI release could be shown with all tissue with cold ischaemic times of 6 hours or more , and that no totally successful way was found to fully preserve islet function after this time.

The consequence of these findings is that one has to exercise extreme care in the evaluation and selection of human pancreatic

tissue for both experimental and possible transplantation studies. Pancreatic tissue has been shown to differ in IRI content by as much as 2,000 % in its initial IRI content . In addition the failure to demonstrate the viability of 25% of these pancreas even under the controlled conditions of this study must raise grounds for concern . These findings serve to reinforce the aims of this thesis in trying to establish criteria predictive of successful islet isolation .

The development of a periperfusion system measuring the dynamic aspects of IRI release in response to alterations in glucose concentration allowed us to define the parameters against which to test the remaining endocrine function following the disaggregation procedures proposed.

Several areas of the digestion procedures have been critically examined . From these findings it was concluded that the use of the McIlwain tissue chopper , intra-venous distention and digestion with a fairly high level of the enzyme or enzymes exhibiting only collagenolytic activity (K_{AV} 0.13 to K_{AV} 0.35 , Figure 6) together with a trypsin inhibitor appeared to offer considerable advantages both in terms of the degree of tissue disaggregation and the apparent viability of the cells and cell aggregates released .

We are aware of the report by Traverso (1978) that a commercially available collagenase preparation (purified by affinity chromatography) was totally ineffective in digesting canine pancreatic fragments . I find it difficult to understand or explain this discrepancy . In this study the collagenolytic

components of clostridial collagenase was the sole factor necessary in achieving disaggregation of the human and as Chapter 5 shows was equally effective in dealing with the canine pancreas. It may be that our use of a more concentrated enzyme preparation could partly explain this difference in results or that their use of a different separation technique resulted in a less effective enzyme preparation. One further explanation may be that as Traverso (1978) demonstrates, the use of purified collagenase fails to activate endogenous enzyme activity which suggests that a measure of 'controlled' autolysis may be a necessary factor in their disaggregation procedure.

Following disaggregation with pure collagenase an average of 69 % of the pancreatic tissue weight was recovered. Islet tissue would appear to have been preserved for no significant difference was found in the IRI content of the disaggregated tissue (Table 16).

Finally periperfusion procedures were used to establish the viability of islet tissue following disaggregation .

A total of 12 periperfusions on disaggregated pancreas were carried out (Table 17). One periperfusion failed to exhibit any potentiated IRI release in response to elevated glucose suggesting that the tissue was or had become non-viable. In the remaining 11 periperfusions sustained potentiated IRI release was demonstrable in 7 periperfusions (67%) compared to 75% of periperfusions of chopped pancreas (Table 12).

The conclusion must be therefore that the disaggregation procedures outlined are capable of providing a tissue preparation which has been shown to be viability at least in the short term.

Provided that criteria for assessing islet function are met the disaggregation procedures described are capable of providing viable islet tissue enabling its application in transplantation studies to be carried out.

It remains to be established however whether this type of unseparated pancreatic disaggregate can be safely implanted or whether further islet separation techniques are needed to produce a 'safe' islet preparation.

CHAPTER FIVE

CANINE AUTO-IMPLANTATION OF UNSEPARATED DISAGGREGATE

5.1 Introduction

It was decided to use the canine model to investigate whether the disaggregation technique (chapter 4) was equally effective when dealing with the dog pancreas .

If this was so then it was also intended to use the dog model to investigate whether this type of unseparated pancreatic preparation could be safely infused and to evaluate its effect in restoring carbohydrate control.

It can also be argued that the outcome of attempts to implant this type of preparation in the canine model may be predictive of outcome following human implantation.

5.2 Experimental Procedures

Three groups of outbred mongrels were used of between 10 and 20 kg in weight .

Group I

Seventeen dogs were used to establish normal fasting levels of plasma glucose , insulin and glucagon. In addition fasting IVGTT's were carried out under sodium pentobarbitone anaesthesia following cannulation of the jugular vein and injection (as a 50% dextrose solution) of a bolus of 500 mg glucose per kg body weight (Bewick 1976). Blood samples were collected into sodium flouride tubes for glucose and lithium heparin tubes containing 10,000 KI units aprotinin for insulin and glucagon estimations. The supernatant was separated by centrifugation and stored at - 20°C as collected .

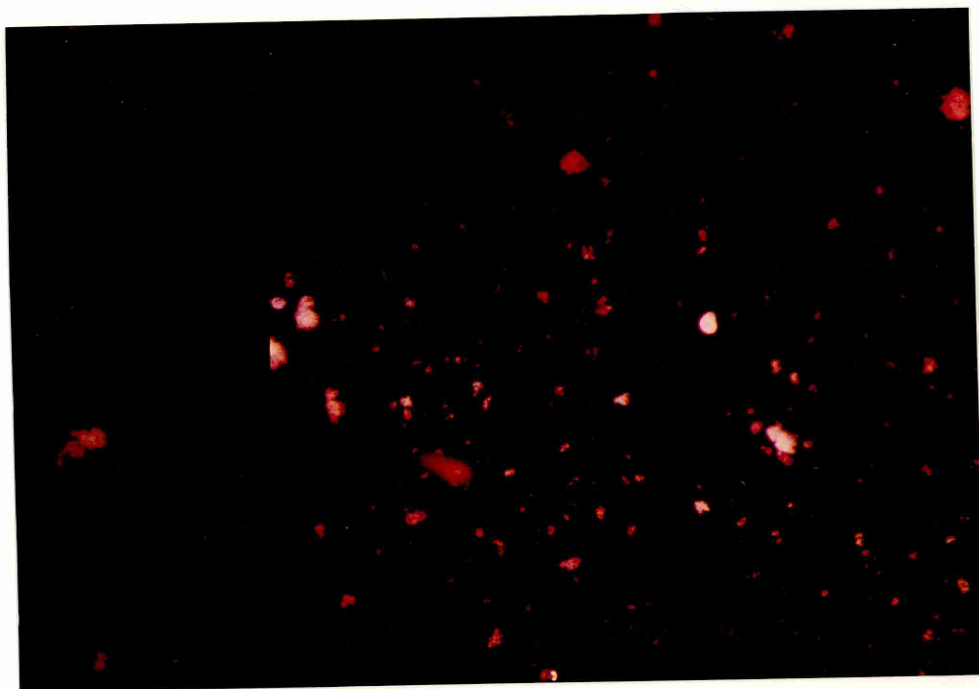
Group II

Eight dogs underwent total pancreatectomy . Post-operative daily fasting plasma insulin and glucose levels were measured and the dogs weight was closely monitored. All pancreatectomized dogs were given 6 Pancrex V forte tablets (Pabryn Ltd.) before meals.

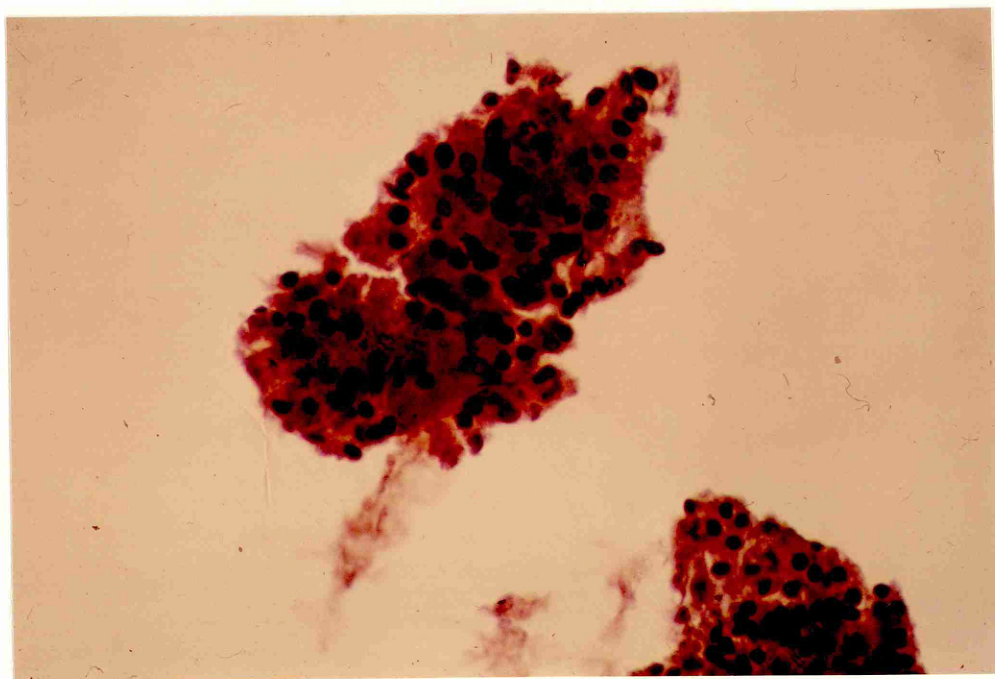
Group III

Nine dogs were subjected to total pancreatectomy . The excised pancreas was disrupted by intra-venous distention , chopped using the Mc Ilwain tissue chopper and digested following the procedures outlined in chapter 4 . The dispersed pancreatic tissue was harvested and washed three times (to avoid introducing free insulin and enzymes contained in the supernatant) by centrifugation at 500 g for 5 minutes intervals. The disaggregate was resuspended in between 100 and 150 ml of RPMI 1640 culture media . This mass was then infused over a ten minute period into a branch of the superior mesenteric vein of one dog and into the splenic artery in the remaining eight dogs between 90 and 120 minutes following pancreatectomy . No attempt was made to interrupt the venous effluent from the spleen and therefore prevent the passage of cells into the portal circulation .

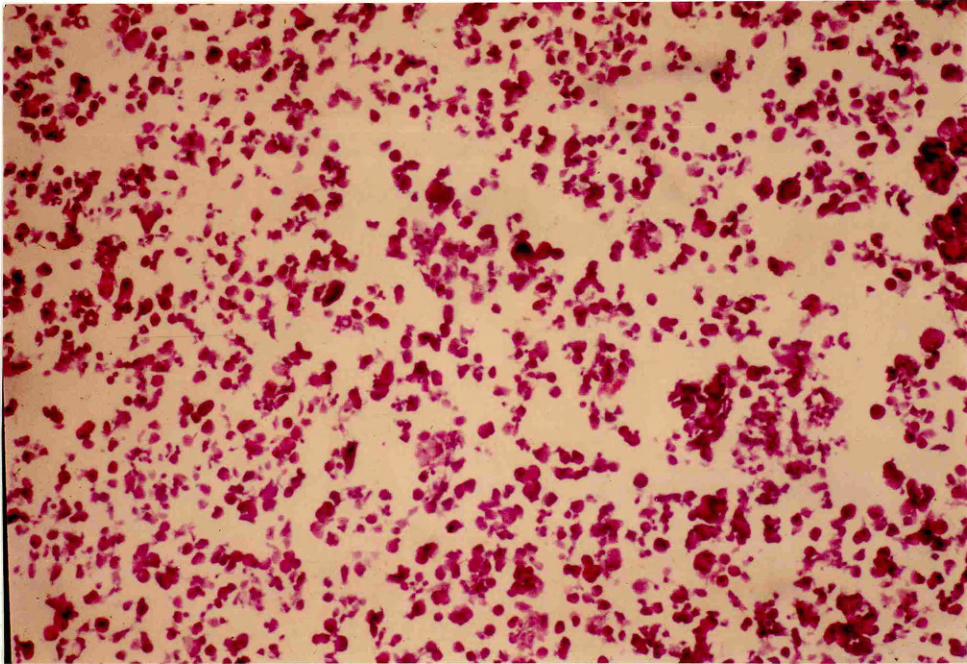
Portal vein pressure was measured using an intra-portal catheter connected by a manometer line to a high fidelity pressure transducer (Model SE 4.82) and monitored using an multichannel recorder (Model SF 4001) , both supplied by Electro - Medical Instruments Ltd..



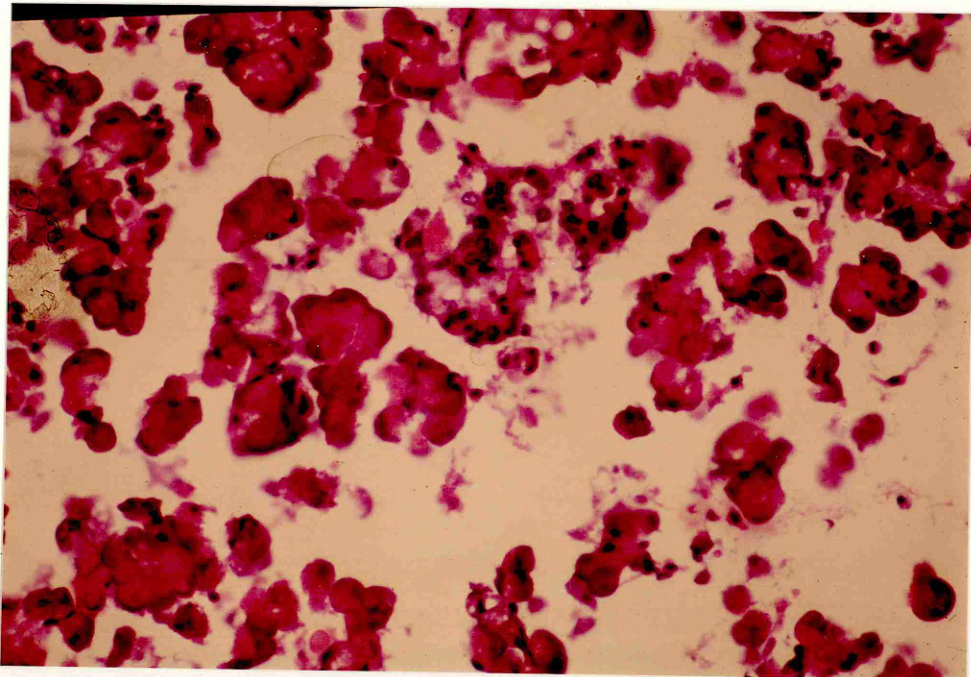
Pancreatic disaggregate - incidental light
Magnification X 10
PHOTOGRAPH 15



Isolated Islets
Haemotoxylin & Eosin Magnification X 250
PHOTOGRAPH 16



Disaggregated human pancreas
Haematoxylin & Eosin Magnification X 20
PHOTOGRAPH 17



Disaggregated human pancreas
Haematoxylin & Eosin Magnification X 200
PHOTOGRAPH 18

4.3 RECOVERY OF HUMAN PANCREATIC TISSUE AND ENDOCRINE FUNCTION

In twelve experiments weighed random aliquots of the chopped pancreatic tissue were subjected to enzymatic disaggregation exactly as described above .

Complete disaggregation occurred in each case and the harvested tissue was washed , weighed and the tissue insulin content was estimated as described (Appendix II).

These results are presented below in Table 16.

RECOVERY OF PANCREATIC TISSUE AND INSULIN CONTENT

Experiment No.	Original wt. of tissue gm.	Wt. of Disaggregate gm.	% Tissue Recovery	Insulin content of disaggregate ng/g/tissue
1	5.156	3.916	75.9%	204,602
2	4.927	3.947	79.9%	30,000
3	1.731	1.123	64.9%	90,000
4	1.436	0.995	69.4%	107,388
5	1.848	1.442	76.9%	76,000
6	1.662	0.988	59.4%	98,604
7	2.814	1.557	55.3%	184,000
8	2.499	1.660	66.5%	31,000
9	2.637	1.854	70.0%	640,000
10	7.635	5.865	76.8%	18,500
11	4.085	3.212	78.6%	26,480
12	4.162	2.448	59.8%	135,530
Mean	3.383	2.416	69.4%	136,842

TABLE 16

Thus on average we recovered 69.4% of the tissue by weight with a range of 55.3 % to 79.9 % recovery over the 12 samples used . The mean recovery of the insulin content of this disaggregated tissue was found to average 136,842 ng/g/tissue which was not significantly different $T = 23$ using the Whitney Mann rank order correlation coefficient) from the value obtained

GLUCOSE CHALLENGES CARRIED OUT ON ENZYMATICALLY DISAGGREGATED TISSUE

IRI Release ng / min / g tissue

Time (mins)	Perfusion												Mean
	1	2	3	4	5	6	7	8	9	10	11	12	
2.8 mMol Glucose	2	31.6	185.5	500	-	-	142.0	52.5	500	-	116	34.0	229.0
	4	27.4	-	429	500	118.4	47.7	-	-	-	-	-	226.5
	6	16.0	74.8	283	51.6	-	60.5	38.1	-	20.6	52.5	30.4	69.7
	8	18.5	64.7	199	44.3	166.9	53.5	35.5	217.7	19.5	47.5	-	76.7
	10	22.5	63.3	329	48.7	-	46.3	39.9	-	28.3	43.2	32.6	72.6
	12	13.8	43.7	484	39.3	142.5	62.8	34.3	113.6	29.4	38.9	-	108.5
	14	12.3	-	283	21.5	129.3	-	28.0	-	20.9	42.7	36.1	87.1
	16	14.3	32.6	235	30.3	68.6	51.9	23.0	47.0	18.5	34.1	-	62.0
	18	18.5	33.9	290	38.2	60.0	54.8	23.4	-	18.5	35.8	40.3	61.3
	20	18.5	-	180	24.0	60.0	62.8	31.8	62.5	23.5	30.9	-	50.6
	22	13.7	26.5	133	28.5	52.8	70.7	27.1	-	16.5	23.4	12.8	40.6
	24	11.8	-	145	30.3	42.6	52.0	24.5	59.2	18.3	31.2	-	44.7
16.8 mMol Glucose	26	9.6	27.8	133	-	43.7	26.6	23.5	71.6	8.3	28.6	10.6	38.4
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	62	7.0	-	133	44.7	-	-	36.7	-	29.6	-	-	50.2
	64	8.7	-	-	30.3	-	-	33.8	75.6	-	-	17.5	33.2
	66	11.5	-	112	20.7	-	-	27.9	-	-	21.2	-	38.7
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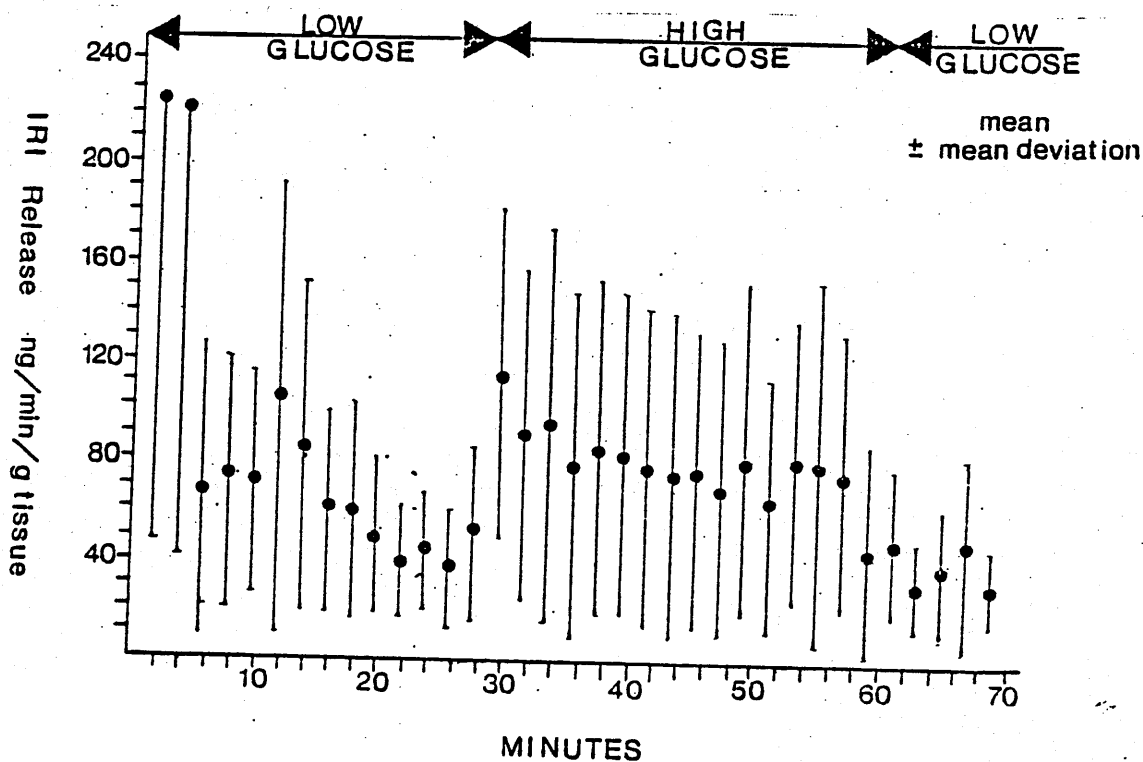


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5.1 Introduction

It was decided to use the canine model to investigate whether the disaggregation technique (chapter 4) was equally effective when dealing with the dog pancreas .

If this was so then it was also intended to use the dog model to investigate whether this type of unseparated pancreatic preparation could be safely infused and to evaluate its effect in restoring carbohydrate control.

It can also be argued that the outcome of attempts to implant this type of preparation in the canine model may be predictive of outcome following human implantation.

5.2 Experimental Procedures

Three groups of outbred mongrels were used of between 10 and 20 kg in weight .

Group I

Seventeen dogs were used to establish normal fasting levels of plasma glucose , insulin and glucagon. In addition fasting IVGTT's were carried out under sodium pentobarbitone anaesthesia following cannulation of the jugular vein and injection (as a 50% dextrose solution) of a bolus of 500 mg glucose per kg body weight (Bewick 1976). Blood samples were collected into sodium flouride tubes for glucose and lithium heparin tubes containing 10,000 KI units aprotinin for insulin and glucagon estimations. The supernatant was separated by centrifugation and stored at - 20°C as collected .

GLUCOSE CHALLENGE ON DISAGGREGATED CANINE PANCREAS

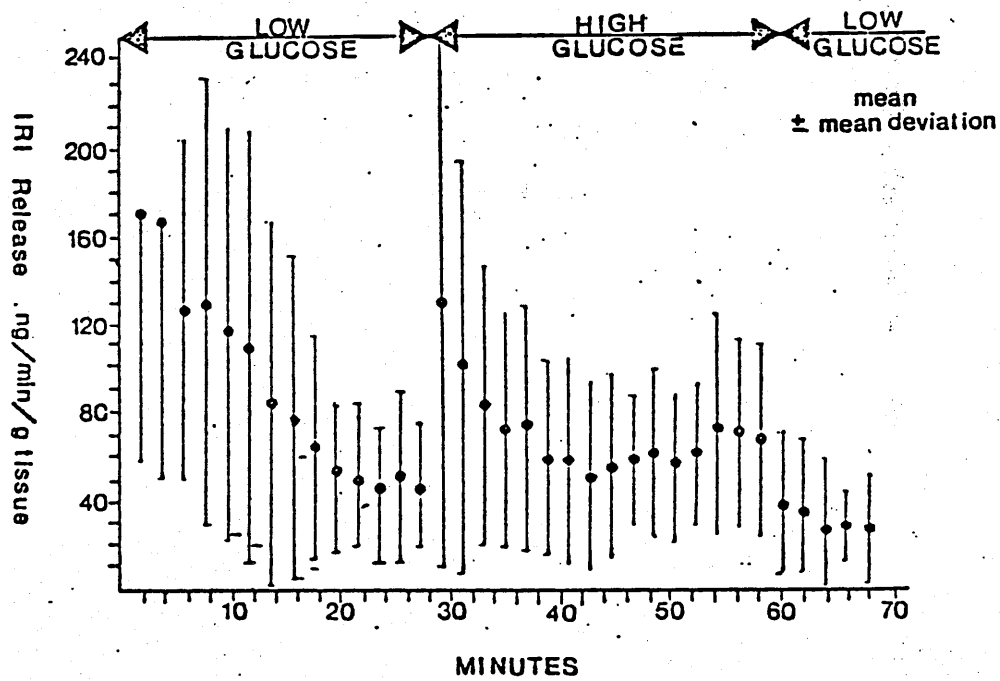


FIGURE 10

Group II

Eight dogs underwent total pancreatectomy . Post-operative daily fasting plasma insulin and glucose levels were measured and the dogs weight was closely monitored. All pancreatectomized dogs were given 6 Pancrex V forte tablets (Pabryn Ltd.) before meals.

Group III

Nine dogs were subjected to total pancreatectomy . The excised pancreas was disrupted by intra-venous distention , chopped using the Mc Ilwain tissue chopper and digested following the procedures outlined in chapter 4 . The dispersed pancreatic tissue was harvested and washed three times (to avoid introducing free insulin and enzymes contained in the supernatant) by centrifugation at 500 g for 5 minutes intervals. The disaggregate was resuspended in between 100 and 150 ml of RPMI 1640 culture media . This mass was then infused over a ten minute period into a branch of the superior mesenteric vein of one dog and into the splenic artery in the remaining eight dogs between 90 and 120 minutes following pancreatectomy . No attempt was made to interrupt the venous effluent from the spleen and therefore prevent the passage of cells into the portal circulation .

Portal vein pressure was measured using an intra-portal catheter connected by a manometer line to a high fidelity pressure transducer (Model SE 4.82) and monitored using an multichannel recorder (Model SF 4001) , both supplied by Electro - Medical Instruments Ltd..

IVGTT's on Normal Dogs

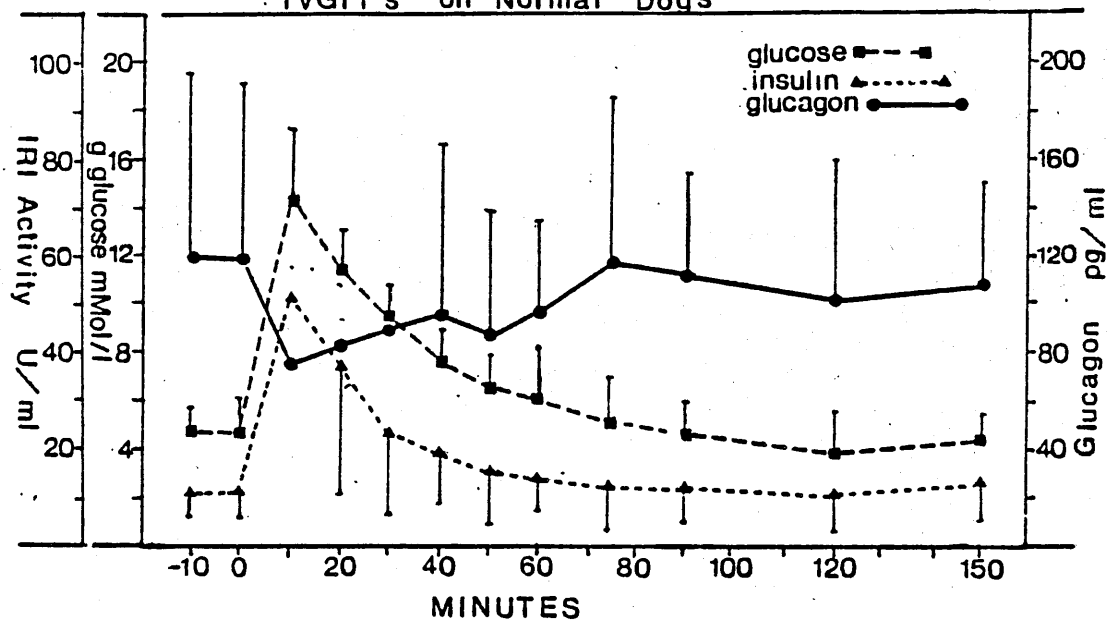


FIGURE 9

DAILY PLASMA GLUCOSE LEVELS M MOL / L

DOG	Days following implantation											
	1	2	3	4	5	6	7	8	9	10	12	
AA1	21.3	24.8	28.0	*								
AA2	21.8	-	-	26.3	27.4	25.6	30.2	-	31.4	-	35.6	*
AA4	23.5	23.5	21.8	-	-	40.3	*					
AA21	17.9	-	-	23.5	25.8	-	-	*				
AA22	21.3	-	-	21.8	23.5	-	24.6	*				
AA26	20.2	22.4	23.5	-	-	33.6	35.8	-	24.9	*		
AA27	17.4	22.4	20.2	35.8	*							
AA28	16.0	20.7	21.3	-	-	28.0	42.0	*				
MEAN	19.9	22.8	21.8	26.9	25.6	31.9	32.5	-	28.1	-	35.6	
S.D.	2.6	1.5	3.8	6.3	2.0	6.6	6.6	-	-	-	-	

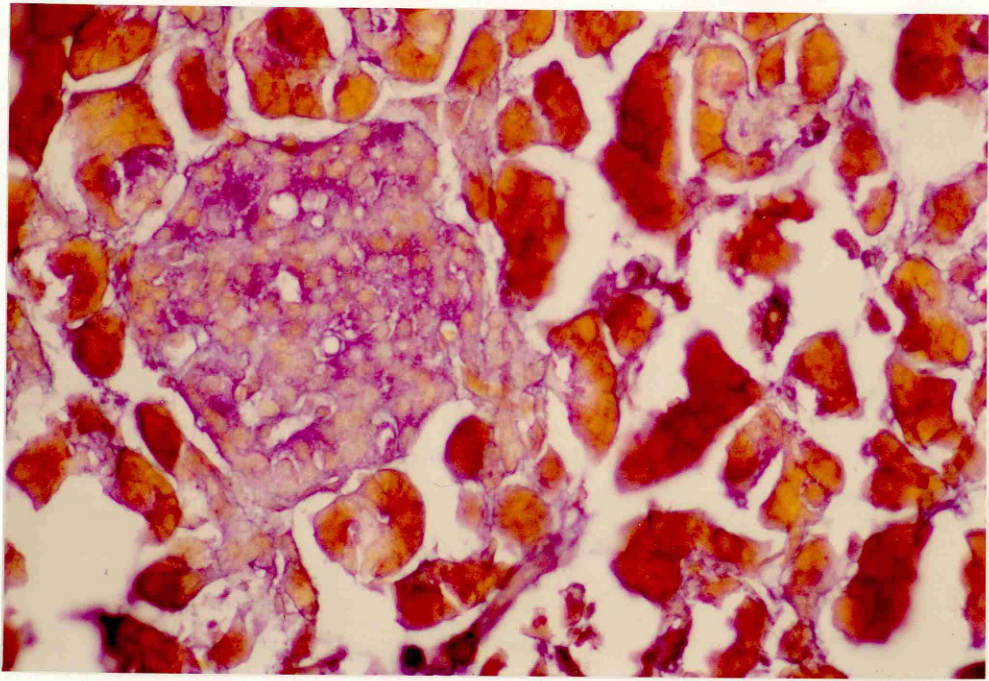
(* = died)

TABLE 18

SURVIVAL AND WEIGHT LOSS OF DEPANCREATISED DOGS

DOG	Plasma Glucose Before Death	Survival Days	Pre-Op Wt. (Kg)	Wt. at Death (Kg)	Daily Wt. Loss (Kg)
AA1	28.0	3	13.0	10.8	0.73
AA2	35.62	12	12.0	8.5	0.29
AA4	40.32	6	10.0	7.8	0.37
AA21	25.76	7	20.0	15.5	0.64
AA22	24.62	7	15.0	10.5	0.64
AA26	24.62	9	16.0	13.0	0.33
AA27	35.84	4	15.0	10.5	1.13
AA28	42.0	7	9.5	7.0	0.36
MEAN	32.09	6.9	13.3	10.5	0.56

TABLE 19



Venous Distention of the Canine Pancreas
Aldehyde - Fuchsin Magnification X 250
PHOTOGRAPH 19

never recovered.

In the eight dogs which underwent implantation through the splenic artery the portal vein pressure was measured on four occasions (Table 20) .

PORTAL VEIN PRESSURE (mm Hg)

Dog	Pre-infusion pressure	Maximum pressure recorded	Post-infusion (20 mins)
AA 25	6	12	7
AA 41	6	15	8
AA 42	11	31	9
AA 43	7	17	8
Mean	7.5	18.8	8
S.D.	2.4	8.4	0.8

TABLE 20

All four dogs exhibited a transient and relatively mild degree of portal hypertension . It was considered unlikely that this level and duration of increased portal pressure would affect the subsequent recovery of these dogs and portal pressure monitoring was discontinued in the rest of this series.

The post-operative course in these eight dogs was complicated in that treatment was needed to correct the tendency towards severe hypoglycaemia and hypokalaemia .These values together with the estimated IRI activity are presented in table 21 .

The most obvious feature of these results are the initial high IRI activity measured in the first hour following implantation .It may be that as in the periperfusion studies (chapter 4) these could be false high estimates due to factors released from the tissue but this was not investigated . What is

PLASMA GLUCOSE AND IRI ACTIVITY FOLLOWING IMPLANTATION

	Plasma Glucose Levels (m Mol/ L)			Plasma Insulin Levels μ U units / ml		
	Mean	S.D	n	Mean	S.D	n
Pre-Operative	5.03	0.35	7	12.8	8.2	7
Pre-Infusion	10.27	3.3	8	3.0	2.6	4
Post-Infusion (mins)						
0 - 30	8.89	3.98	4	484.0	326.0	4
30 - 60	9.46	5.29	5	162.7	36.3	5
60 - 90	7.32	3.32	5	41.7	16.3	4
90 - 120	5.88	2.16	5	39.8	11.3	5
120 - 150	5.66	3.84	4	35.9	12.7	4
150 - 180	3.16	1.3	5	16.2	6.2	4
180 - 210	1.25	0.16	4	15.0	6.6	4
210 - 240	-	-	-	11.3	9.6	3
240 - 270	All animals receiving parenteral dextrose (see text)			10.5	7.1	5
270 - 300				8.5	10.6	3
330 - 360				7.9	4.4	6

TABLE 21

SURVIVAL FOLLOWING PANCREATIC TISSUE IMPLANTATION

DOG	Survival (days)	Outcome	Cause of Death
BB1	0	Died	No obvious cause
BB2	0	Died	Massive internal Haemorrhage
AA42	10	Despatched	Severely jaundiced
AA43	12	Despatched	Weight loss ,infected, generally unwell
AA45	0	Died	No obvious cause
AA46	1	Died	Massive internal Haemorrhage
3B3	5	Despatched	Jaundiced,gener- ally unwell
3B4	0	Died	Massive internal Haemorrhage

TABLE 22

clear is that this apparent high IRI activity although immunologically active in the radio-immunoassay would appear to be in a biologically inactive form i.e. pro-insulin or one of its derivatives . In the normal animal levels of insulin such as this would initiate an extremely rapid fall in the blood glucose levels which clearly did not occur .

This was clearly not the case two to three hours later . In the presence of falling IRI levels of activity the plasma glucose level fell precipitatusly . In the case of three dogs ,to below the level which is known to produce hypoglycaemic coma. In two dogs the rate of decline in plasma glucose was estimated at three hours following implantation to be 9.13 and 11.17 m Mol / L over a 30 minute period , equivalent to a loss of between 325 and 400 mg of glucose per kg body weight per hour.

In an attempt to prevent these events over the immediate post-operative period , between between 100 and 250 g of a 20% dextrose solution and between 1.5 and 4.2 g of potassium chloride was slowly infused between three and six hours following implantation .

The recovery and survival of these eight dogs proved disappointing (Table 22).

Four dogs died during the first night, one died during the second night and the remaining three were depatched for humane reasons at 5 , 10 and 12 days , each with grossly elevated plasma glucose levels although each had evidence of insulin secretion (Table 23).

Necropsy was carried out on all animals. Of the five dogs which died within 24 hours the spleen in all cases was contracted

POST-OPERATIVE DAILY BLOOD SUGAR AND INSULIN LEVELS

Days post- implantation	Plasma Glucose				Plasma Insulin			
	Levels M Mol/L				Levels μ U/ml			
	AA42	AA43	AA46	BB3	AA42	AA43	AA46	BB3
1	18.8	12.9	6.3	5.0	4	10.5	11	1
2	-	15.7	*	16.2	-	4	*	-
3	-	-		-	-	5		23
4	18.6	-		-	6	-		-
5	20.2	-		-	5	5		36
6	21.3	24.1		*	7	4		*
7	22.2	24.6			12.5	5.5		
8	22.0	26.9			21	9.5		
9	20.8	22.0			12	8		
10	-	22.0			-	42		
11	24.0	*			21	*		
12	24.3				26			

(* = died)

TABLE 23

POST-OPERATIVE BLOOD COAGULATION FACTORS

Time	Prothrombin Time n=4		Kaolin-cephalin Time n=4		Platelet Count X 10	
	Mean	S.D	Mean	S.D.	Mean	S.D.
Pre- Implantation	9	1.2 (range 8-10)	20.5	2.1 (range 18-23)	232	68 (range 179-329)
Post-implan- tation (mins)						
60	15	2.6 (range 12-18)	37.3	7.7 (range 30-48)	55	13 (range 37-67)
120	16.5	3.7 (range 12-20)	36.3	7.8 (range 29-46)	94	40 (range 54-147)
180	17	4.2 (range 14-20)	42.0	5.7 (range 38-46)	84	44 (range 53-115)
240	18	5.7 (range 14-22)	42.5	10.5 (range 28-57)	93	59 (range 51-134)
300	20.5	3.5 (range 18-23)	34.5	13.4 (range 25-44)	65	16 (range 54-77)

TABLE 24

to between 1/2 and 3/4 of its pre-operative size but this is a well recognized consequence of the agonal state.

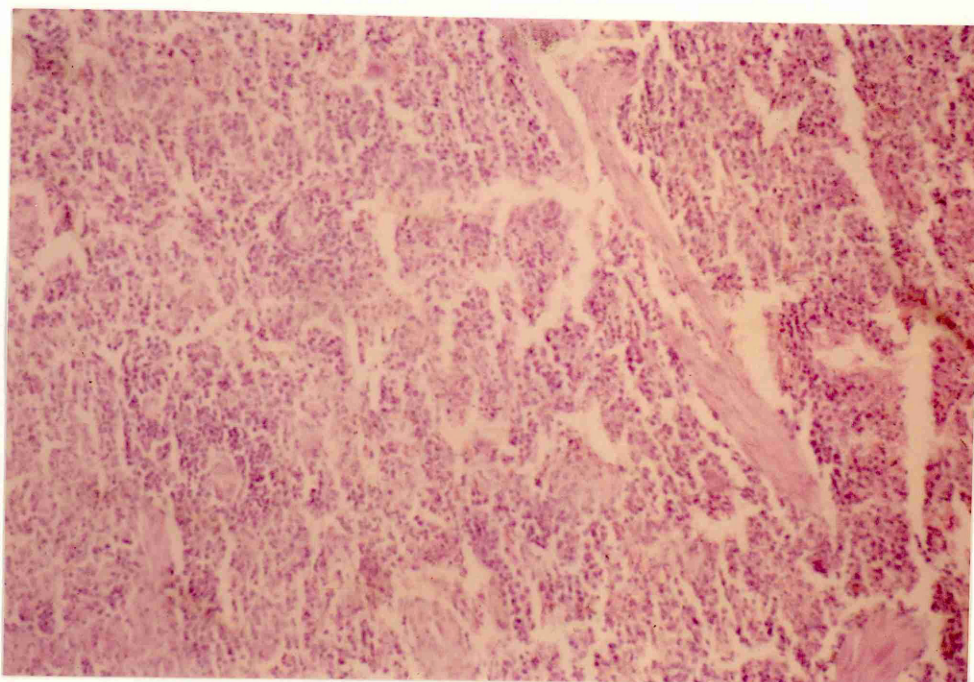
There was no macroscopic evidence of occlusion to either large or small arteries of the spleen . Histological examination of the spleen revealed a relatively normal tissue morphology (Photograph 21). The one surprising finding was the almost complete absence of pancreatic fragments and only 3 out of 20 histological sections provided evidence of pancreatic tissue remaining within the vasculature of the spleen (Photograph 22). The conclusion must be drawn that either these pancreatic fragments failed to lodge within the arterial and sinusoidal network or failed to survive in this site.

No abnormality was noted in any other organ with the exception of the liver which in all cases was cream coloured and friable. Microscopic examination revealed that severe cellular necrosis had occurred (Photographs 23 & 24) the cause of which remains unknown .

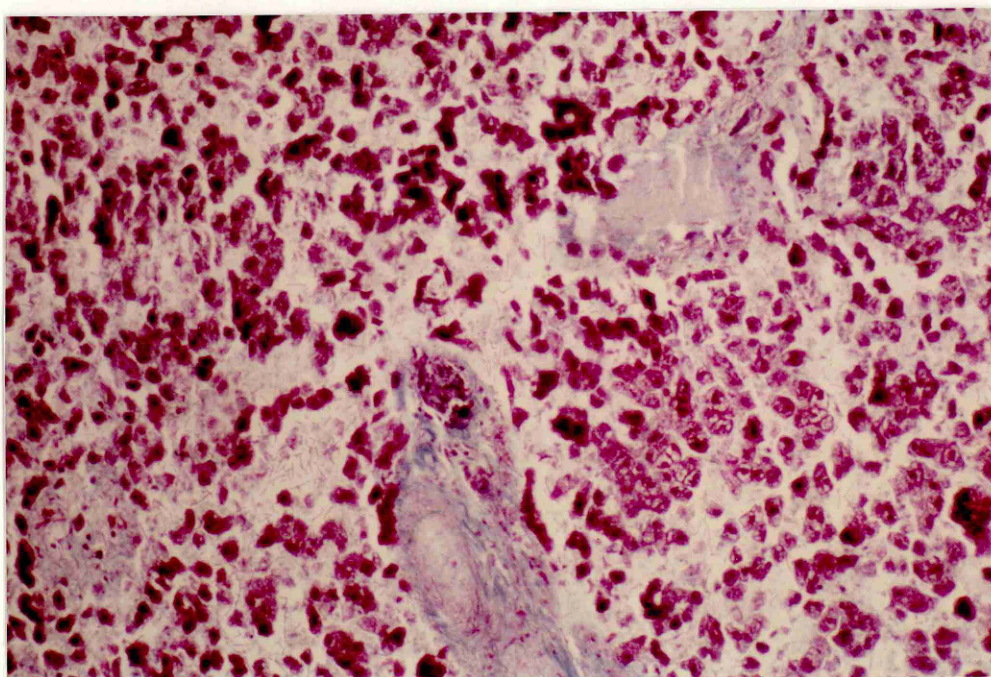
Of more serious concern was the finding that despite care in ensuring haemeostasis following pancreatectomy three dogs died as a result of massive haemorrhage arising from the site of the excised pancreas.

The death of the first dog and the protracted bleeding from the site of wound closure led us to suspect that a clotting disorder similar to intravascular coagulation had occurred.

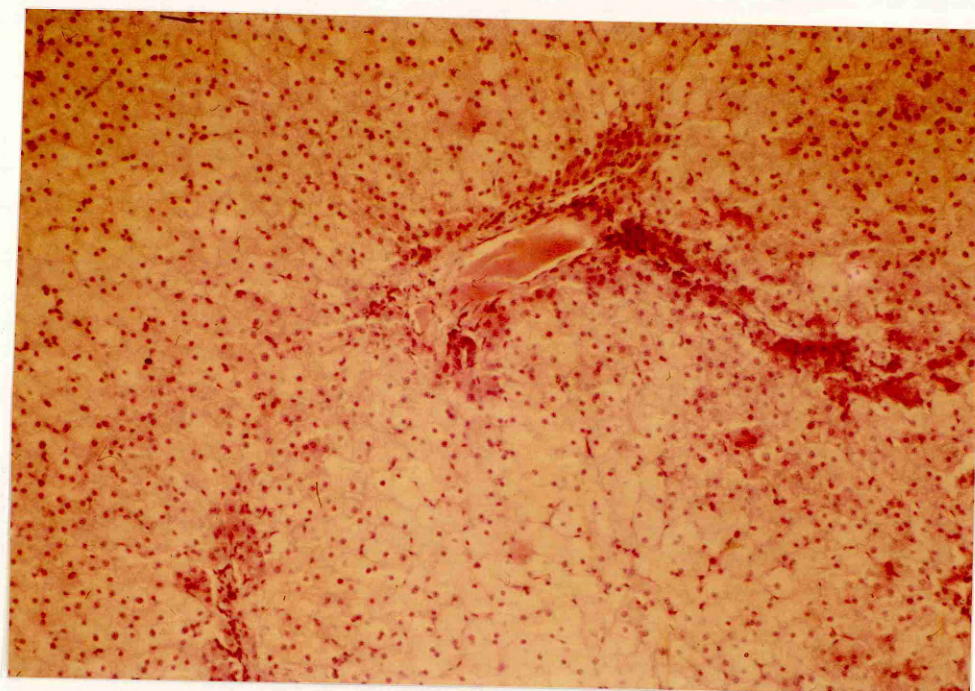
This problem was investigated in four subsequent dogs by measuring the prothrombin (PT) time , kaolin-cephalin (KCT) time and the platelet count both before and following implantation. These results are presented in Table 24 .



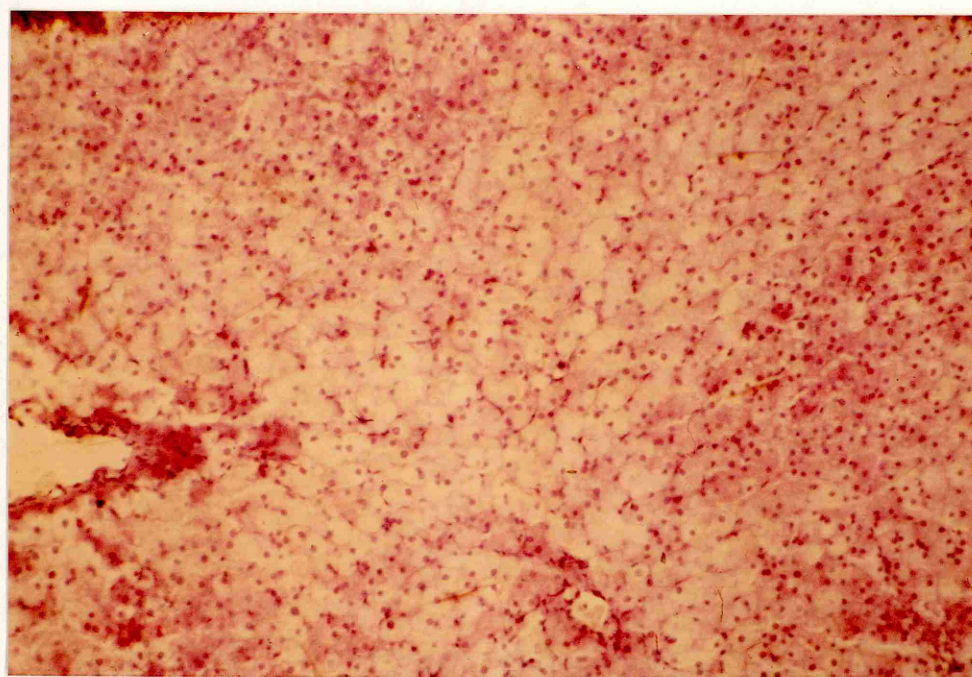
Canine Spleen 20 Hours After Implantation
Haematoxylin & Eosin Magnification X 25
PHOTOGRAPH 20



Canine Spleen 20 Hours After Implantation
Showing Tissue Occluding Artery
Mallory Magnification X 100
PHOTOGRAPH 21



Hepatic Cellular Necrosis
20 Hours After Implantation
Haematoxylin & Eosin Magnification X 25
PHOTOGRAPH 22



Hepatic Cellular Damage
20 Hours After Implantation
Haematoxylin & Eosin Magnification X 50
PHOTOGRAPH 23

Haemostasis can be considered as an ongoing process which is dependent upon vascular integrity, platelet count and the coagulation plasma proteins as well as the inhibitors and thrombolytic factors which maintain the balance of this system. The process of coagulation can be accelerated by either vascular damage or by thrombogenic substances within the bloodstream which over-ride the ability of inhibitors to maintain haemostasis.

When intravascular coagulation is accelerated there is a decline in some of these clotting factors (notably platelets and factors I , II and XII) which can lead to severe and uncontrolled bleeding.

As can be seen (Table 24) the implantation of pancreatic tissue was followed by an increase in PT from 9 to in excess of 15 seconds an increase of KCT from 20.5 to between 34.5 and 42.5 secs. and a fall in platelet counts from 232×10^6 to between 55 and 94×10^6 platelets / ml , which were sustained over at least six hours. This coagulation pattern is diagnostic of disseminated intravascular coagulation (DIC) having occurred although these studies do not of course elucidate the cause.

Further studies of this severe haematological dysfunction were carried out in conjunction with two associates and with the clinical haematology department of Guy's Hospital. This experimental evidence showing that components of the implanted pancreatic tissue was directly responsible for the onset of D.I.C. and suggestive evidence as to the cause of this dysfunction have been published (Miller 1983) .

Of the four dogs which survived in excess of one day two had blood sugar levels within the normal range on the first post-

operative day . One of these dogs died during the second postoperative night. Subsequently all dogs had elevated levels of plasma glucose not significantly different from the pancreatectomized animals (Table 18 , page 121).

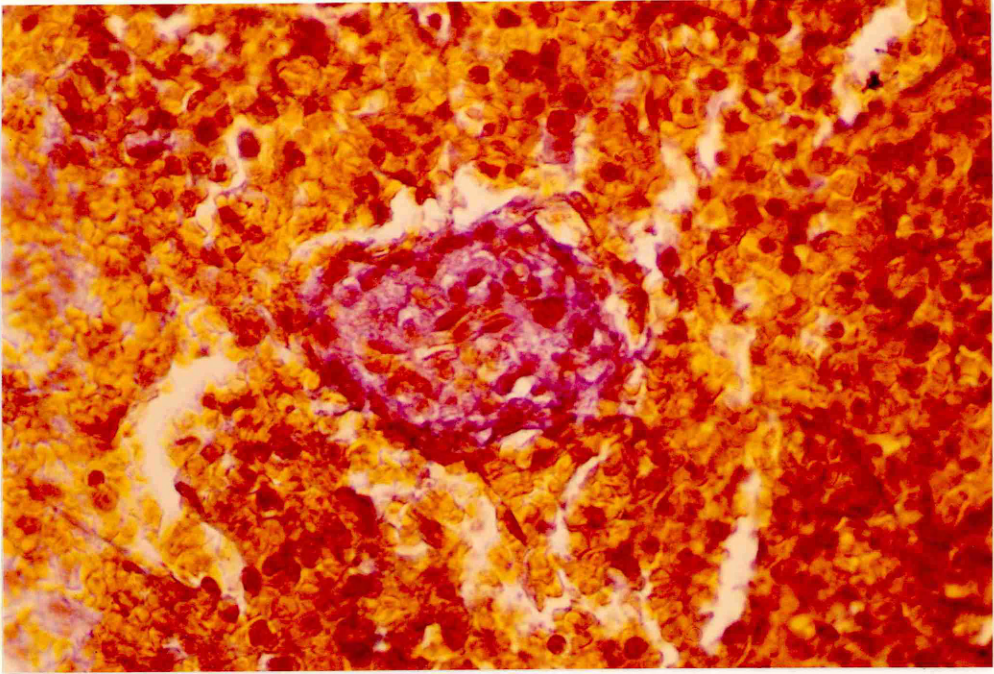
In each of these dogs increasing levels of fasting IRI activity were noted to in excess of normal fasting levels. What this means in terms of the level of restored pancreatic endocrine function remains unclear as we have no figures for IRI activity in control animals with a corresponding level of hyperglycaemia. Nevertheless it does indicate that some islets must have survived. Confirmation of this was provided by the histological demonstration of a few islet like structures and islet cells which stained with aldehyde fuchsin (photographs 24 & 25) .

Summary and Conclusions

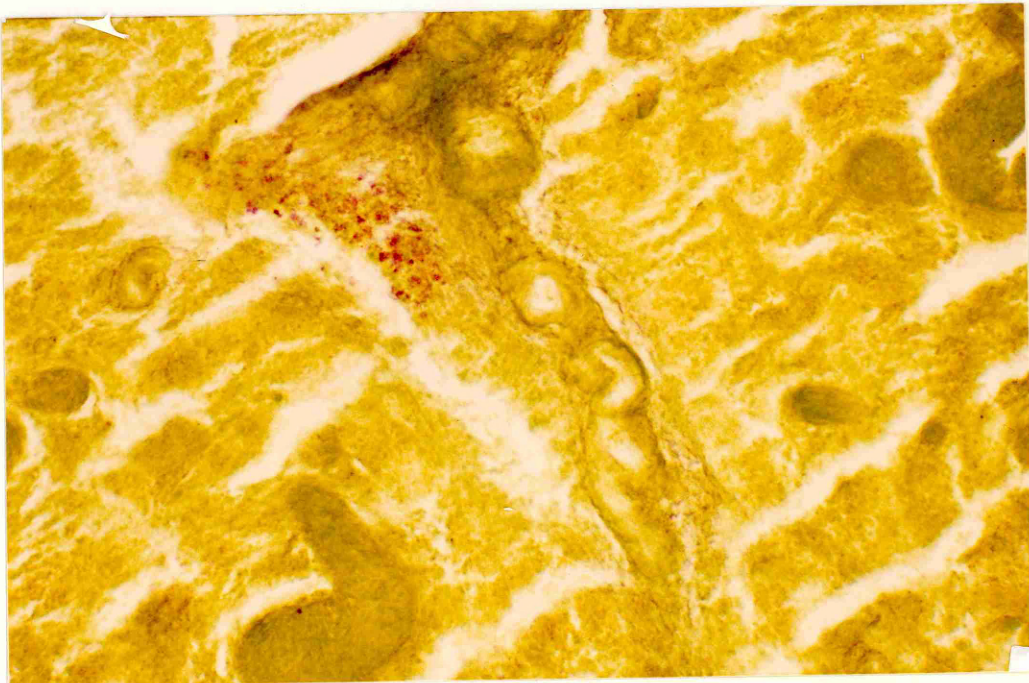
Attempts have been made to evaluate the quantitative and qualitative endocrine function contained within the (non-separated) pancreatic disaggregate. In excess of 60% of the pancreatic tissue volume and 50% of the insulin secreting capacity was recovered following disaggregation .

Implantation of this volume of tissue into the portal vein was followed by a severe and fatal portal hypertension.

Of the other sites available the spleen would appear to offer certain advantages in that as in the normal animal insulin is secreted directly into the portal circulation . In addition splenic implantation following subcapsular (Kretshmer 1977) and interstitial (Mirkovitch 1976) implantation have proved successful in the canine model. Reported instances of



Intra-Splenic Islet
Aldehyde Fuchsin Magnification X 400
PHOTOGRAPH 24



Intra-Splenic Aldehyde Fuchsin
Positive Cells
Aldehyde Fuchsin Magnification X 100
PHOTOGRAPH 25

subcapsular haematomas, intrasplenic necrosis ,intrasplenic cavitation and subcapsular perforations (DuToit 1982) suggest that this approach could not be applied in attempts at clinical implantation . Even without these reported complications there is a certain reluctance on the part of the regional transplant team to even consider splenic capsular rupture (personal communication).

For this reason implantation in the canine model was carried out by infusion into the splenic artery . There were no apparent signs of damage to the splenic parenchyma . Considerable signs of cellular trauma were however shown to have occurred within the liver .It is possible that this trauma could have resulted from the limited degree of portal hypertension or the severe fluctuations in blood glucose during the first 20 hours following implantation. A more likely explanation is that cellular components of the implanted tissue may have produced this effect. The total splenic outflow drains directly into the liver and the effect of certain enzymes e.g. pronase (Mills 1969) have been shown to exert a marked selective cytotoxic effect upon hepatocytes.

Implantation of the dispersed pancreatic disaggregate resulted in a marked increase in plasma IRI activity which was followed by the rapid disappearance of plasma glucose from the systemic circulation . Parenteral glucose was used in an attempt to prevent critically low levels of blood sugar but it is thought that hypoglycaemic coma may still have occurred and may have caused the death of the two dogs in which no other cause of death could be found.

Implantation was also shown to be followed by severe intravascular coagulation which is thought to have caused the massive bleeding diathesis which resulted in the death of three dogs. This problem has been reported and considered by two other groups who suggest that prophylactic treatment using aprotinin (a trypsin / kallikrein inhibitor) and systematic heparination can abrogate this haematological disorder . This was not investigated as evidence suggests that although the risk may be diminished it is not removed . Indeed , uncontrolled bleeding which eventually proved fatal has been reported in two patients who received aprotinin and anticoagulation therapy (Mehigan 1980 and Toledo-Pereyra 1983).

Despite infusion of in excess of 60% of the original pancreatic tissue volume there was an apparent paucity of pancreatic tissue fragments within the spleen suggesting that very little tissue had survived in this site. In the three dogs which survived more than two days ,elevated levels of plasma glucose only slightly less than those of the pancreatectomized control group were recorded until death. In each dog however , increasing plasma IRI activity to between 26 and 42 μ U/ml were recorded . This would be in agreement with the studies of Mirkovitch (1976) which first indicated that endocrine function may take many days to recover sufficiently to restore normoglycaemic control .

In this study there is another possible explanation. In the normal animal the liver plays a major role both in carbohydrate control and insulin degradation by enzymes conveniently labelled

as 'insulinases '. It is not known whether the observed hepatic damage and the probable loss of these enzymes may have affected these results.

It may be that the site of implantation was inappropriate. Kretschmer (1977) found implantation into the splenic artery to be ineffective in restoring normoglycaemia in three dogs with a mean survival of 13 days and a fasting level of hyperglycaemia at death of 24.6 m Mol / L . It is possible that as Kretschmer (1977) reported sub capsular implantation would have been more effective but the aim of this study was to eventually attempt to apply this technique in man and rupture of the splenic capsule could provide considerably risk.

In conclusion our experience indicates that implantation of this type of dispersed pancreatic tissue into the splenic vasculature was ineffective in influencing the diabetic state. In addition it represented an extremely hazardous procedure with serious life threatening consequences .

If islet cell implantation is to become a reality then it is our opinion that as in the rat model ,purified islets represent the only safe preparation and this must therefore be a prerequisite before attempts at clinical implantation can be justified.

Further results to be presented and continuing research into islet cell implantation have been carried out in the belief that a relatively pure islets cell preparation is essential if the aims of a 'safe and effective ' cure for the disease is to become a reality.

CHAPTER SIX

TECHNIQUES OF ISLET ISOLATION

6.1 Introduction

From the results presented in the preceding chapter on auto-implantation in dogs it is obvious that implantation of unseparated disaggregate is ill advised . There is no evidence to suggest that isolated islet implantation in man may be less safe than the experimental studies in rodents would indicate.

We therefore need to look at methods of isolating and implanting pure islet preparations . Experimental evidence in rodents suggests that pure islet preparations invoke no significant degree of hepatic damage or exert any thrombogenic influence upon the blood clotting mechanism following implantation .

In order to arrive at any measure of islet cell enrichment it is necessary to exploit factors which lead to either selective survival or selective harvesting of the cells of interest. As in animal studies several approaches are possible .Selective survival of islets and death of exocrine tissue in culture either with (Matas 1976) or without (Reemtsma 1980) non-endocrine tissue toxins are a possibility . Alternative approaches relate to differences in the physical properties of the desired cell population i.e. differences in density or cell size (Scharp 1974), electrical membrane potential (Matthews 1969) and surface properties such as membrane adhesive factors (Chick 1977).

Alternatively some investigators have further fractionated

islets into individual cells. Velocity sedimentation ,density centrifugation (Piperleers 1981) and flourescent activated cell sorting techniques (Nielsen 1982) have been used to purify these individual cell lines and even reaggregation of the individual cells into islet like structures has been reported (Downing 1979). The yield of islet cells following disaggregation into single cell suspensions has however been extremely low which does not suggest that this approach could be applied to providing sufficient cells for transplantation studies. In addition there may be valid physiological reasons (i.e. the paracrine relationship between islet cells , page 13) why an intact islet preparation is to be preferred.

Thus many techniques of cell separation have been used. Of the more obvious and readily available methods it is intended to investigate whether culture techniques or differences in density or size , or adhesive properties can be applied to arrive at a viable purified islet preparation.

6.2 Separation Techniques

6.2.1 Culture of dispersed pancreatic tissue

Standard tissue culture techniques were used to examine the survival of non-separated pancreatic disaggreate.

Between 100 and 200 μ l of the washed dispersate was placed in Multiwell tissue culture dishes (Flow Laboratories) and 4 ml of RPMI 1640 containing 10% foetal calf serum and 1% antibiotic-antimycotic solution was added. The wells were kept within a modular incubator (Flow Laboratories) which was gassed with 5% CO₂ in air under maximum humidity. The cultures were nourished by

CULTURED NON-SEPARATED PANCREATIC TISSUE

IRI Content of the Culture Media μ U / ml					
Days in culture					
1	2	4	6	8	
1,830	970	228	101	31	
2,000	460	112	34	8	
127	78	90	30	3	
,225	76	57	15	5	
510	115	81	16	3	
360	125	46	9	3	
1,700	410	107	42	11	
1,700	1,290	360	42	9	
410	95	53	11	5	
610	200	10	2	0	
Mean	947	382	114	30	8
S.D.	757	424	104	28	8

TABLE 25

exchanging two thirds of the culture media on the first and every subsequent 2 days of culture.

Culture of unseparated tissue was carried out on 10 separate occasions and in each case the B cell function would seem to be lost after a few days (Table 25).

The amylase level of the culture media was 9.45 ± 3.6 on day 1 and fell to 4.37 ± 2.1 and 1.15 ± 0.85 on days 2 and 4 respectively indicating the failure of exocrine components to survive.

Before discussing the measured IRI activity it is perhaps appropriate to briefly consider the criteria we are using to assess the survival of endocrine tissue under culture conditions.

In Chapter 4 it was argued that the only valid indicator of islet viability was a continuous IRI release under low glucose stimulation and a potentiated and sustained IRI release in response to elevated glucose levels under controlled conditions as in the periperfusion studies .

Several factors may combine to make this type of study inappropriate when dealing with cultured tissue . Islet tissue may attach to the surfaces of culture vessels and may be difficult to recover intact . Of more serious concern is the evidence that suggests that islet cells become degranulated during culture (to be discussed more fully in Chapter 7) and consequently the normal pattern of primary and secondary IRI secretion may not correlate directly with the volume or the viability of the cultured endocrine tissue.

In view of these problems in assessing the viability of islet tissue in culture it was considered that for the purpose of

ADDITION OF APROTININ TO NON-SEPARATED DIGEST

Aprotinin Conc.K.I.U.	IRI Concentration Of Culture Media				
	Day 1	Day 2	Day 4	Day 6	Day 8
0	743 ± 435	365 ± 194	89 ± 58	37 ± 14	11 ± 2
1,000	1193 ± 633	247 ± 145	106 ± 60	40 ± 19	16 ± 6
2,500	1307 ± 531	517 ± 180	299 ± 133	156 ± 72	57 ± 39
5,000	1243 ± 421	515 ± 269	224 ± 88	123 ± 66	35 ± 15
10,000	1405 ± 615	385 ± 91	166 ± 93	111 ± 49	48 ± 41
20,000	1227 ± 393	422 ± 101	303 ± 273	88 ± 89	42 ± 35

(mean ± S.D., n=5)

TABLE 26

the following experiments , continuing release of IRI into the tissue culture media would provide a direct indication that the islet tissue was surviving . In terms of the viability of islet tissue or its normal responsiveness to secretagogues it is of course necessary to investigate the dynamics of IRI release in response to high and low glucose levels. For this reason the only valid assumption we can make at this time is that the rapid disappearance of IRI activity indicates that the tissue has become non-viable. Conversely as we are replacing 2/3 of the culture media every two days an increase in IRI levels to in excess of 1/3 of the previous measurement indicates continued secretion and therefore continued cell viability .

Following culture of the non-separated disaggregate (Table 25) there was a 60% loss in IRI activity on day two followed by a 70%, 73.7% and 74.2% decline on days 4,6 and 8 .This failure to demonstrate any de novo IRI secretion suggests that islets were failing to survive under these culture conditions. The addition of aprotinin to the culture media resulted in an increased measurement of IRI activity over the period of culture (Table 26) which appeared maximum at a concentration of above 2,500 KIU/ml . It therefore seems likely that the rapid loss of IRI activity is due to released proteolytic activity in the culture media , the effect of which could be only partially prevented by the use of this proteolytic inhibitor.

These results suggested that culture of non-separated pancreatic disaggregate cannot be of use in arriving at sufficient islets to reverse diabetes following implantation.

SEDIMENTATION VELOCITY OF DISAGGREGATED PANCREAS

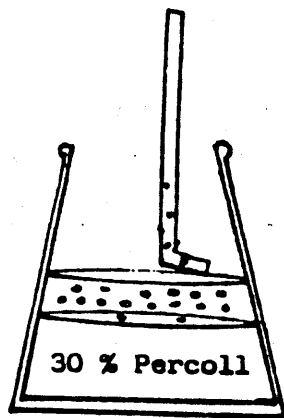


FIGURE 11

DENSITY CENTRIFUGATION OF DISAGGREGATED PANCREAS

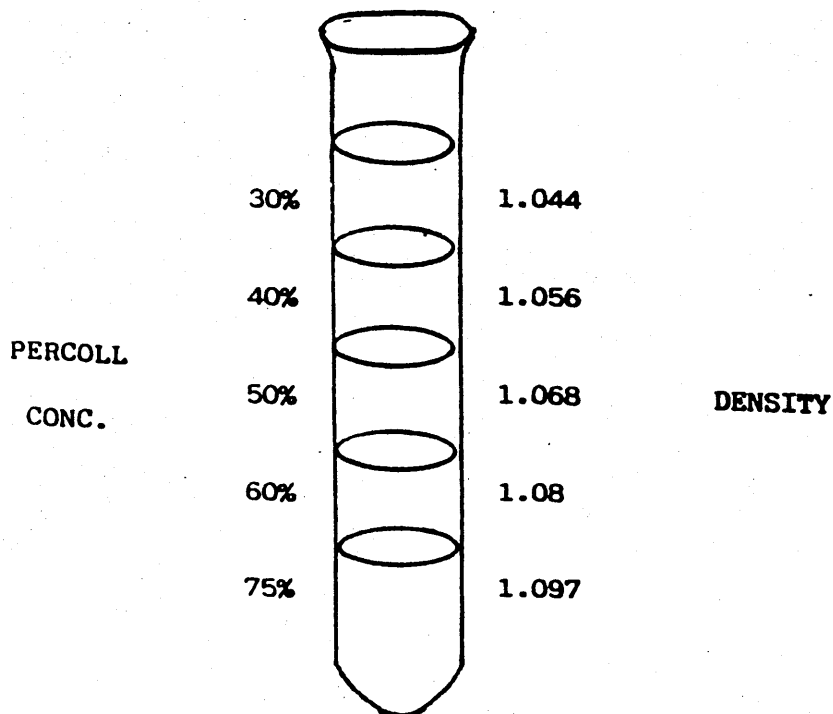


FIGURE 12

6.2.2 Density Centrifugation and Velocity Sedimentation

Many variations exist in the techniques available to separate cells on the grounds of either differences in size or density (for a comprehensive review see Pretlow 1974). All however are dependent upon an approximation to Stokes Law ;

$$V = \frac{d (P_1 - P_2)}{18 n} \times g$$

where V= sedimentation rate d= diameter of the particle
P₁= particle density P₂= density of the liquid
n= viscosity of liquid g= gravitational force

On purely theoretical grounds velocity sedimentation and density separation are not entirely independent procedures. Although from the above equation it can be seen that sedimentation separates cells on the basis of both cell density and cell size. For most cell lines , size has been shown to be inversely proportional to cell density (Cavalier-Smith 1978). Thus , sedimentation velocity separates cells mostly on the basis of particle size.

In the case of density centrifugation the sedimentation rate will tend towards zero as the particle density approaches the density of the media . Therefore density centrifugation (or more accurately in the case of this study , isopycnic sedimentation) separates cells on the basis of cell density .

Velocity sedimentation (although more usually performed on single cell preparations) was carried out by layering 2ml aliquots of dispersed pancreatic tissue over 25 ml of an iso-osmotic Percoll solution (see below) with a density of 1.045 (Buitrago 1977) and a surface area of 40 sq. cm (Figure 11). At the end of 15 minutes the upper tissue layer and sedimented

PERCOLL CENTRIFUGATION

Percoll Interface	% Tissue Weight at each Interface						Mean
	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5	Exp.6	
100-75%	7.8	2.7	0	2.8	2.7	0	2.70
60-75%	20.4	8.9	14.5	17.7	8.9	1.4	11.97
50-60%	15.4	9.5	5.2	15.4	9.5	1.7	9.45
40-50%	34.1	54.1	11.2	14.9	54.1	22.6	31.83
30-40%	9.1	11.9	16.4	16.8	11.9	26.6	15.45
0-30%	13.2	12.9	52.3	32.5	12.9	48.7	28.75

TABLE 27

PERCOLL CENTRIFUGATION

Percoll Interface	% Insulin Content at each Interface						Mean
	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5	Exp.6	
100-75%	11.0	1.4	0	0	0.5	0	2.15
60-75%	21.4	10.6	0.4	16.7	8.1	8.6	10.97
50-60%	11.8	14.3	1.8	24.2	11.0	12.6	12.62
40-50%	28.0	45.9	22.6	28.2	64.2	41.7	38.40
30-40%	9.3	12.6	26.6	10.1	8.9	14.6	13.68
0-30%	18.5	15.2	48.8	20.9	7.8	22.5	22.30

TABLE 28

PERCOLL CENTRIFUGATION

Percoll Interface	Insulin Content at each Interface (ng/g/tissue)						Mean
	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5	Exp.6	
100-75%	70,805	11,344	-	-	7,976	-	30,042
60-75%	93,084	47,590	2,533	91,340	39,200	61,429	55,879
50-60%	126,871	118,387	26,670	155,133	53,234	74,118	92,407
40-50%	326,255	148,778	167,587	185,600	51,112	184,513	177,307
30-40%	264,057	122,226	134,589	58,000	32,212	54,884	110,996
0-30%	201,067	74,198	76,642	61,418	26,043	46,201	80,928

TABLE 29

tissue was removed , weighed and assayed for insulin content .

Measurement of the insulin content of the sedimented and non-sedimented tissue showed that while a fairly pure islet preparation was obtained , the yield of insulin containing tissue recovered in the sedimented tissue was extremely low , being 3.8 ± 2.14 % of the total tissue insulin content . This low yield suggests that this technique would be unable to yield sufficient islets to warrant attempts at transplantation and therefore no further studies were carried out .

Density Centrifugation ,

After adjustment of the Percoll with X10 concentrated Hanks solution to achieve an osmolality of 300 mOsm/kg , further dilutions using iso-osmotic Hanks solution were made to produce computed densities of between 1.044 and 1.097 . A discontinuous gradient was constructed by layering 3mls of each density within a 20 ml conical tube (figure 12) . Following the preparation of the gradients 2 mls of washed digest was added and the tubes were centrifuged at 2,000 g for 20 minutes . Following centrifugation the tissue was seen to have separated at each interface into 6 discrete bands . These layers were carefully collected , weighed and either assayed for insulin content or placed into culture .

The tissue weight and insulin content (expressed as a percentage) of the tissue from each of these fractions are presented in Tables 27 and 28 .

The tissue fragments can be seen to have settled over the total range of densities used , with the greatest tissue volume (75%) being concentrated in the less dense layers . This of

CULTURE OF PERCOLL SEPARATED TISSUE

Percoll Interface	IRI Concentration Of Culture Media				
	1 Day	2 Days	4Days	6 Days	8 Days
0 - 30 %	782 ± 706	196 ± 94	50 ± 21	20 ± 18	8 ± 3
30 - 40 %	575 ± 240	202 ± 65	71 ± 32	31 ± 25	13 ± 6
40 - 50 %	718 ± 329	314 ± 212	193 ± 111	137 ± 109	68 ± 56
50 - 60 %	693 ± 234	271 ± 38	186 ± 39	130 ± 20	113 ± 33
60 - 75 %	79 ± 80	16 ± 19	11 ± 15	2 ± 2	2 ± 3
75 - 100 %	8 ± 9	4 ± 6	3 ± 3	2 ± 3	0 ± 0

(Mean ± S.D. ; n = 6)

TABLE 30

course means that (unlike density centrifugation of rodent islets) these human islet preparations will be grossly contaminated by non-endocrine components .

The total insulin content at each density (Table 29) showed that IRI containing tissue could be recovered at each of the interfaces used and that whichever layer was selected a marked loss in endocrine tissue was inevitable.

The insulin content of the tissue sedimenting at each interface shows however that a degree of islet enrichment had occurred in some fractions.

The most IRI enriched tissue content (177,307 ng/g/tissue) was shown to sediment at a density of between 1.056 and 1.068 . This layer contained 38.4% of the total insulin containing tissue and 31.83 % of the total tissue volume .

The tissue at each interface was cultured using the procedures outlined above . IRI release into the culture media is shown in Table 30 .

The rapid fall in IRI activity of tissue settling at densities of below 1.056 and above 1.08 suggests that it was or became non-viable during the period of culture . In contrast it can be seen that although a decrease in IRI activity in the culture media was measured , culture of the tissue at the 1.056/1.068 and 1.068/1.08 interfaces resulted in a level of IRI release in excess of 30% of the previous measurement . The conclusion must therefore be that continued IRI secretion occurred and therefore at least a proportion of the endocrine tissue was and remained viable during the period of culture . It is tempting to speculate that the components present in non-

Insulin Content prior to and following filtration			
	Insulin content prior to filtration ng/g/tissue	Insulin content following filtration ng/g/tissue	Percentage reduction
Exp.1	300,800	195,230	35.1
Exp.2	422,990	307,640	27.3
Exp.3	89,000	42,000	52.8
Exp.4	243,170	77,360	68.4
Exp.5	328,000	130,960	39.9
Exp.6	63,120	44,240	29.9

TABLE 31

STATIC GLUCOSE CHALLENGE (ng per initial gm tissue)			
	Low glucose	High glucose	Low glucose
Exp.1	350	226	225
Exp.2	116	607	235
Exp.3	112	110	25
Exp.4	200	260	120
Exp.5	225	244	116
Exp.6	133	310	125
Mean	189	293	141
S.D.	91	167	78

TABLE 32

separated dispersed pancreatic tissue responsible for the rapid cell death of endocrine tissue during culture (Table 25 , page 135) may have been retained within the less or more dense Percoll layers . This raises the possibility that this type of preparation might be safely implanted .

4.2.3 Cell adhesion

Although the mechanisms involved remain unclear it is known that certain cells will adhere to certain chemo-attractants such as collagen (Postlethwaite 1978) , and fibronectin (Gauss-Muller 1980).

The ability of human islets to preferentially adhere to such a surface was tested by filtering the washed pancreatic disaggregate through a collagen coated 1,000 μ m nylon mesh (as described in Appendix IV) supported within a Sartorius filter vessel (model SM-16-510). It was considered that a reliable estimate of the percentage of insulin containing tissue which adhered to the collagen filters could be obtained indirectly by measuring the insulin content of the disaggregated tissue prior to and following filtering . This showed that on average the filtered tissue insulin content was reduced from a mean of 241,163 to 132,905 ng/g/tissue following filtration (Table 31) indicating that on average 42.2% of the endocrine tissue had been retained upon the filters .

Ethanol extraction of two of these filters confirmed that the insulin containing tissue had been retained on the nylon mesh with comparable IRI content to that of the loss from the filtered tissue .

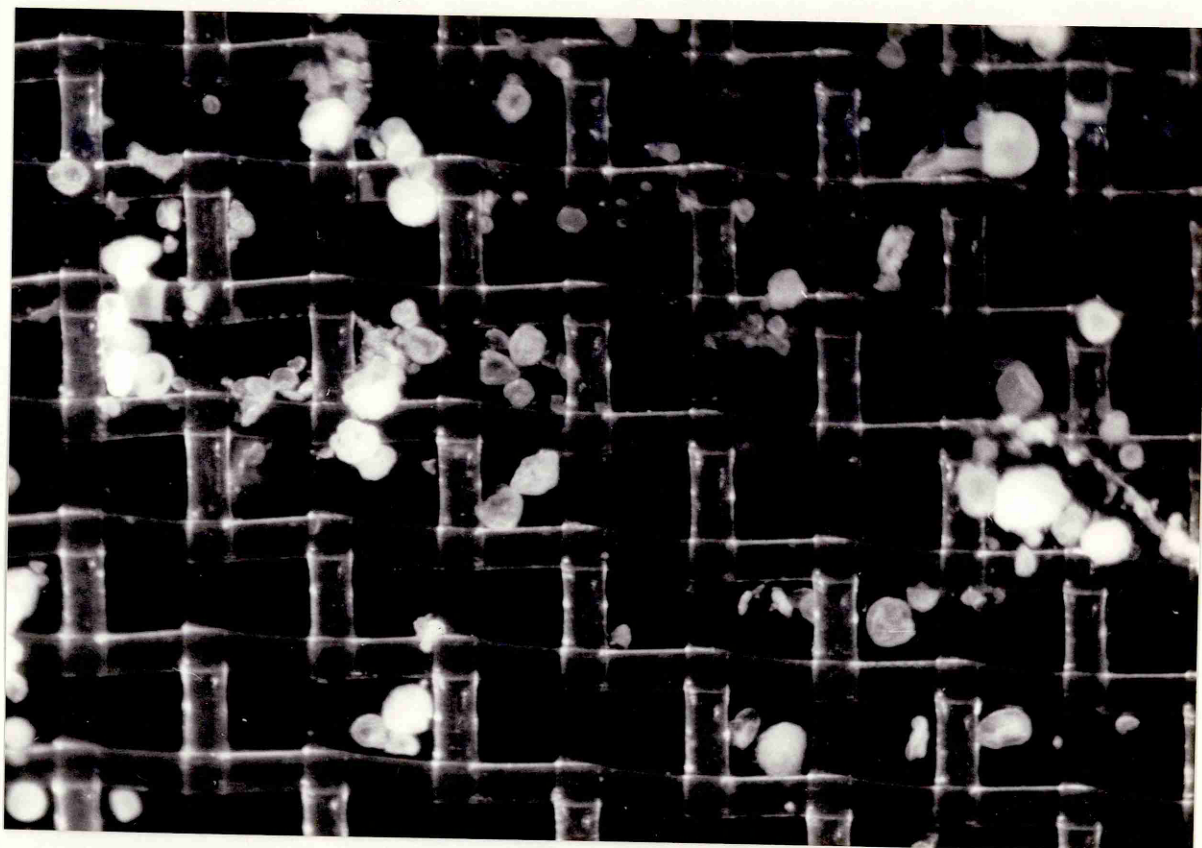
Examination of the coated nylon mesh (Photographs 26 & 27) indicated that an almost pure preparation of islet tissue could be isolated .

The ability of these freshly isolated islet preparation to respond to alterations in glucose concentrations was tested by 3 consecutive 30 minute incubations in the periperfusion solution containing 2.8 , 16.8 and 2.8 mMol/l glucose .

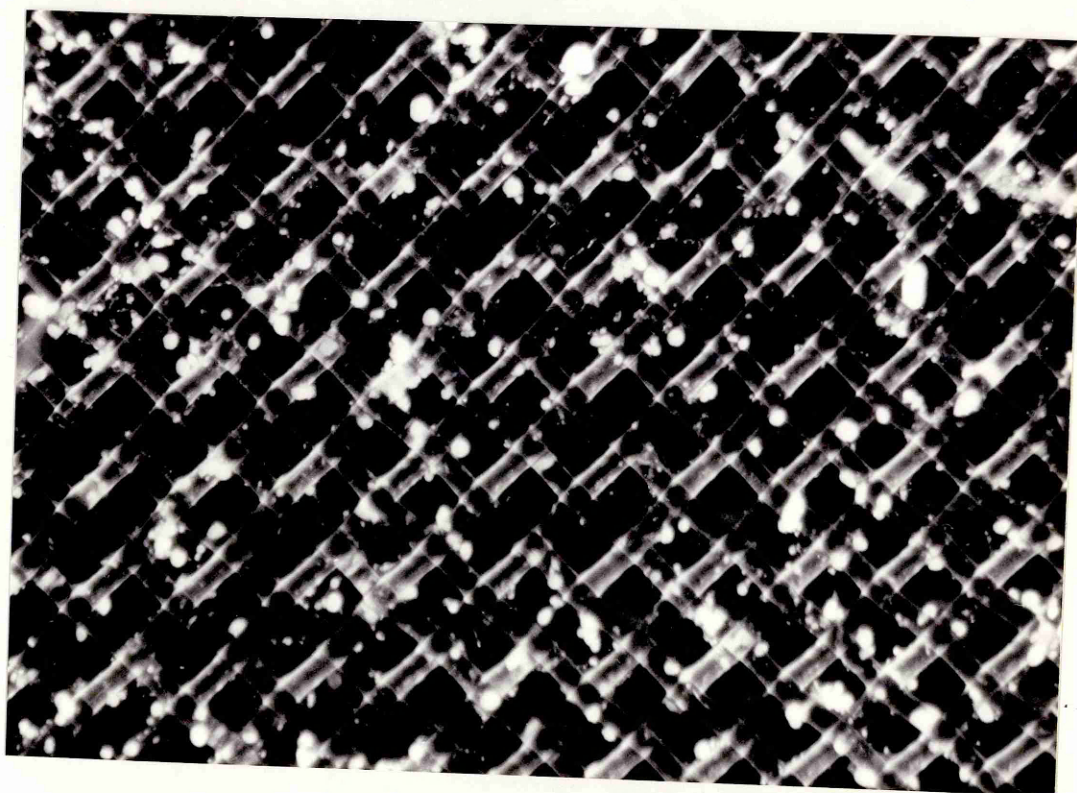
The mean rate of IRI release during each 30 minute incubation was found to increase from 189 to 293 ng per initial g of tissue in response to a stimulatory level of glucose (Table 32) . In only 50% of the purified preparations was there unequivocal evidence of a sustained IRI release which increased in response to high glucose levels . As in the periperfusion studies (Chapter 4) it would seem that not all tissue preparations had retained their normal physiological secretory potential .

In a further series of experiments islets have been removed from the filters by exposure to collagenase and maintained under culture conditions as described previously .Continued IRI release during 8 days of culture (Table 33) suggests that the islets isolated in this manner were capable of surviving under tissue culture conditions .

In summary we have investigated four techniques for harvesting islet preparations . The results suggest that while density centrifugation of dispersed human tissue would appear to have only a marginal effect upon islet cell enrichment ,the continued IRI release into culture of the endocrine tissue



Collagen Coated Filter
Magnification X 20
PHOTOGRAPH 26



Collagen Coated Filters
Magnification X 10
PHOTOGRAPH 27

CULTURE OF COLLAGEN ISOLATED ISLETS

IRI Concentration of Culture Media U / ml					
	Day 1	Day 2	Day 4	Day 6	day 8
	993	353	308	92	152
	857	327	218	133	187
	940	315	169	93	168
	820	255	315	124	208
	1475	480	300	448	178
	700	435	550	296	390
	490	340	310	465	250
	710	750	408	356	292
	425	535	270	238	245
	398	205	126	155	114
	430	510	215	265	295
Mean	749	409	290	242	223
S.D.	323	154	116	137	79

TABLE 33

sedimenting at a density between 1.056 and 1.08 indicates that it was viable and at least a percentage of the islet tissue remained so during the 8 days of culture . Whether this type of cultured preparation could be safely implanted remains unknown however.

In contrast collagen coated nylon mesh filtration allowed the recovery of an almost pure islet preparation . Both direct and indirect measurements of the adherent tissue indicates that approximately 40% of the insulin containing tissue can be recovered from the pancreatic disaggregate using collagen coated filters .

This figure suggests that approximately 25% of the original islet mass remained after the procedures outlined to disaggregate the tissue and arrive at a purified islet preparation.

In terms of islet survival ,static glucose challenges have been used to identify and select those islet preparations

isolated by chemotaxis which were shown to be viable .

Whether the implantation of only 25 % of the islet tissue would be sufficient to fully restore normoglycaemia following implantation remains unknown . Evidence from both segmental whole organ and experimental islet implantation suggests that implantation of a reduced islet volume results in a return to the diabetic state . Thus it would seem likely that we have to consider the possibility of using islet material from more than one donor .

The introduction of culture techniques enabling preservation of the endocrine tissue would appear to hold certain advantages .

It would provide time so that islet function could be assessed and sterility ensured . It may offer advantages in that a short period in culture has been suggested as a prerequisite for successful engraftment in some models (Payne 1979 , Selawny 1978) .

Perhaps the greatest potential benefit of in vitro culture may be in dealing with the allograft tissue phenomena .

The final chapter of this thesis is therefore concerned with briefly considering techniques and attempts to preserve islet morphology and function in vitro and in reporting preliminary experiments at implanting long term cultured islets across a xenogeneic histocompatibility barrier .

CHAPTER SEVEN

CULTURE AND XENOGENEIC IMPLANTATION OF HUMAN ISLETS

7.1 Introduction .

Most early investigators attempting in vitro cultivation of pancreas used classical explant techniques with foetal or neonatal tissue as their starting material.

The first indication that foetal derived islet tissue would survive in vitro was provided by Chen (1954) following watchglass culture of 13-15 day rat foetal tissue. Electron microscopy studies and tinctorial staining of the explanted tissue revealed typical endocrine cytoplasmic granules throughout the 9 days of culture.

Coalson (1956) used the rat diaphragm assay and Dieterge-Lievre (1960) measured the loss of PAS positive granules from co-cultured liver cells to show that the functional activity i.e. insulin and glucagon secretion of the pancreatic explants could be preserved in vitro .

Similar results were obtained with foetal or neonatal pancreatic endocrine tissue from other species. Chick (Black 1954) , neonatal mouse (Wells 1963) and embryonic rat (Zagury 1959) were reported to survive and in some cases continue growth and functional differentiation during culture .

Trowell (1959) was the first to demonstrate that the naturally occurring media used in these cultures could in the most part be replaced by a defined culture media. A wide variety of these often very complex media have subsequently been used , although the addition of a small amount of serum has in all cases

except one (Buitrago 1975) been shown to be necessary for cell survival (Andersson 1977 , Nielsen 1979).

Foetal tissue has since been successfully cultured as explants at the air/gas interface of the media (Hegre 1977) , attached to artificial capillaries (Knazek 1977) and submerged in media in petri dishes (Chick 1975) where under appropriate experimental conditions the endocrine tissue has been encouraged to form monolayers of replicating B cells (Fisher 1977) .

Early studies suggested that islets had essential requirements for survival in vitro . An in vitro dependency upon adrenal secretion in maintaining pancreatic tissue integrity was shown by the work of Zagury (1959) . It is interesting that a similar although sex specific dependency upon adrenal secretion was shown to occur in vivo (Grossman 1969) .

Moskalewski (1965) first showed the importance of the glucose concentration of the medium used . It is now generally agreed that culture in low glucose (below 5.6 mMol) results in a depression of IRI synthesis and release ,and to impaired response of the islet tissue to increased glucose levels. Culture in 15 to 20 mMol glucose leads to extensive degranulation of B cells but may result in nuclear hypertrophy , DNA synthesis and cell division (Lacy 1977) . Andersson has suggested that high stimulatory levels of leucine may substitute in some part for glucose .

Since then many other factors have been suggested to play a supportive role in maintaining islet cell viability in vitro .

These include ions such as calcium (Schatz 1982), vitamins such as nicotinamide (Andersson 1980) and inositol (Clemens 1979) , adenosine (Fussganger 1976) , branched amino acids e.g. leucine but not valine (Nakhooda 1977) , hormones such as somatotrophin (Larsson 1978) , somatostatin (Williams 1980) and glucocorticoids (Andersson 1977) as well as homologous serum (Goldman 1976) and perhaps surprisingly the serum from newly diagnosed diabetics (Nielsen 1977) .

To this must be added the possible effect on islet tissue of one of a number of recently discovered factors shown to be potent stimulators of cell proliferation (for a recent review of these growth factors see Sato 1982).

Thus it can be seen that a bewildering array of techniques and additives have been reported to maintain the morphological and functional integrity and to support the growth of endocrine tissue during culture .

Under appropriate culture conditions , the continued growth and development of human foetal derived endocrine tissue has been reported (Leach 1973) , (Agren 1980) and (Maitland 1980).

In contrast both in vivo and in vitro growth of adult derived islet tissue has been the exception rather than the rule (King 1977) .

Although rare in vivo (LeCompte 1962) , an increased rate of islet cell mitosis has been reported following conditions where chronic elevations of blood glucose occur (Brosky 1975) , concomitant with regeneration of hepatic tissue (LeCompte 1965) and in the presence of certain insulintrophic compounds i.e. following the administration of ACTH and cortisone (Kinash

1954) .

There is general agreement that a degree of adult islet cell replication can be stimulated by increased glucose levels in vitro (Moskalewski 1977) however the mitotic index would appear to be low and we are aware of no cases where a non-transformed permanently dividing (primary) B cell line has occurred .

This means that although growth of adult islets in culture remains an exciting possibility the culture of adult islets must be viewed at present as simply a means of preservation of islet tissue prior to other studies i.e. transplantation.

Survival of adult derived islets in culture have been reported by many investigators (see Sutherland 1981) . Cultured isolated islets have been shown to maintain their ability to both synthesize and secrete insulin (Schatz 1982) . The effect of culture techniques in preserving islet function has been tested further by implantating cultured rat islets into streptozotocin diabetic rats. Scharp (1974) reported a delayed return to normoglycaemia. Nakagawara (1978) and Bretzel (1982) however noted no difference between cultured or fresh islets in the time needed for the restoration of normoglycaemia while Selawry (1978) reported a beneficial effect of short term culture on islets from older rats. This suggests that any changes in endocrine tissue function which may occur during short term culture would appear to be reversible following implantation.

We must remain aware however that in vitro culture introduces a totally artificial environment and that the culture

conditions may induce differences in the physiological response of the cultured islet tissue . For instance many investigators have shown that daily IRI release into the culture media decreases over the first 7 to 10 days of culture although insulin biosynthesis remains unaffected (Schatz 1982) .

From this it follows that IRI release may be considerably reduced during the period of culture .This has been reported even in the presence of a rapidly increasing islet mass (Maitland 1980) . In addition it has been suggested that degradation of IRI activity (Andersson 1977) , adaptation of the cells to high glucose levels (Moskalewski 1977) and feedback inhibition by accumulation of hormones i.e. somatostatin (Turcot-Lemay 1975) may combine in underestimating the remaining endocrine viability and function .

It is recognized that more sensitive techniques (i.e. the incorporation of radio-labelled metabolites into insulin and pro-insulin or periperfusion techniques) are necessary to quantify islet survival . As yet no studies have however been carried out along these lines.

In this study our primary aim at this time was to investigate the possibility of culture as a means of preserving islet tissue and if successful to evaluate the effect of long term culture in reducing the immune tissue rejection phenomena .

7.2 Culture of Human Islets,

Human islets were purified and recovered from the collagen coated filters . Between 50 and 100 islets were introduced as free floating cultures into non-coated multiwell dishes

IRI RELEASE INTO CULTURE

μ U / ml of culture media							
Days	Mean	S.D	n	Days	Mean	S.D	n
3	1720	848	30	30	335	214	10
6	1000	212	30	33	215	75	10
9	935	272	29	36	135	92	10
12	865	238	14	39	210	95	9
15	684	640	14	42	165	80	10
18	515	316	10	45	158	48	10
21	395	280	10	51	175	78	15
24	578	430	10	54	128	64	10
27	375	230	10	60	168	58	9

n = 30

TABLE 34

IRI RELEASE DURING CULTURE

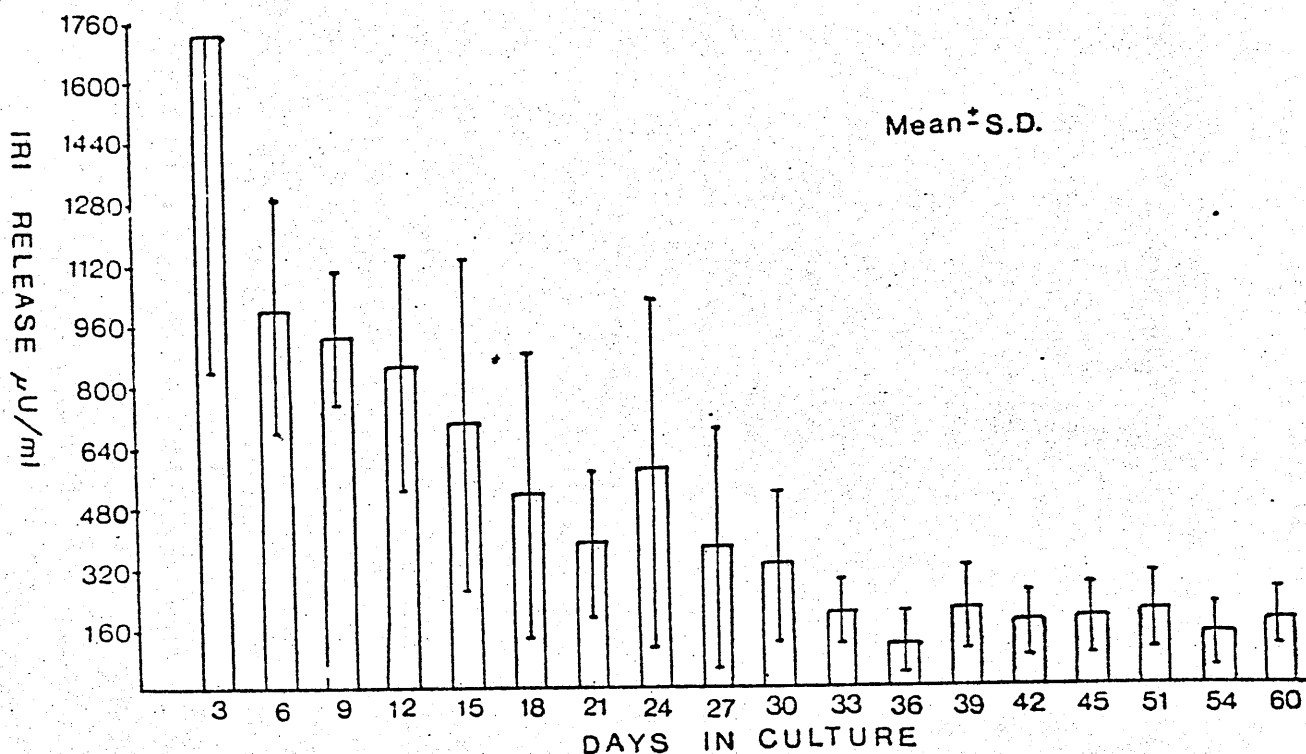


FIGURE 13

containing 4 ml of culture media . The culture media was RPMI 1640 with the addition of 10 % foetal calf serum (Flow Laboratories type 29-101) and 1% antibiotic-antimycotic solution (Gibco-Biocult) . The cultures were maintained in a humidified atmosphere of 95% air and 5% CO . Two thirds of the culture media was exchanged every 3 days by centrifugation of the dishes using plate holders (type M18F , Dynatech) at 500g for 5 mins.

The IRI content of the culture media over a 60 day period is shown in Table 34 and Figure 13 .

Examination of the cultured islets using an inverted microscope provided evidence that islet and cell morphology had been preserved during the period of culture . IRI production rates (IRI released into the media during 3 days culture) decreased progressively over the first 21 days of culture from 1,720 to 395 μ U / ml , decreased to 335 μ U / ml between day 21 and 30 and remained fairly constant from 30 days onwards at a level of 170 μ U / ml .

Although the reasons for the decline in IRI release remain unexamined these results clearly show that a degree of IRI release can be preserved in free floating islet cultures for a period of up to 60 days which demonstrates continuing islet survival .

This allowed us to carry out a pilot study to see whether the immunogenicity of these cultured islets could have been altered by the period of in vitro culture .

7.3 Xeno-implantation of long term cultured islets

With the exception of such procedures as skin sensitization

tests in man there would appear to be no obvious model in which to test the Summerlin hypothesis upon human cultured islets .

Three lines of evidence however are of note and suggested that any loss of immunogenicity of human islets following long term culture could perhaps be investigated even across a discordant xenogeneic barrier .

The long term growth and survival of human islet tissue in athymic immunodeficient nude mice and rats (Usadel 1980) suggests that the only barrier to xenotransplantation is of an immunological nature .

Xenogeneic implants into immunocompetent hosts are usually rejected within a few hours (Shons 1974). Two theories of the pathogenesis of the hyperacute xenograft rejection have been proposed (see Kemp 1978), both of which involve rapid cellular destruction following activation of the complement cascade.

Bretzel (1982) reported that there was no evidence of this hyperacute xenogeneic tissue rejection mechanism following islet implantation even when discordant species were used i.e. pig islets implanted into rats. Both the time of onset of rejection (3 days) and the morphological evidence was apparently similar to the assumed cell mediated rejection phenomena seen when implantation has been carried out across allogeneic barriers. In addition Shumakov (1980) has reported one month survival of cultured human foetal pancreas in immunocompetent rats.

If this is so , then the rat model could be used to evaluate the effect of long term culture on altering the immunogenicity of human islet tissue.

It was decided to follow the protocol initiated by Lafferty (1975) who successfully transplanted thyroid tissue following a period of 26 days in culture .

An experimental study was therefore carried out using a limited number of AC2 rats of between 150 and 200 g in weight. Freshly isolated human islets were implanted into the peri-renal space in four rats . Two rats received human islets which had been maintained in culture for 28 days and which still showed continued IRI release into the culture media. In addition two rats served as controls for normal blood sugar and insulin levels.

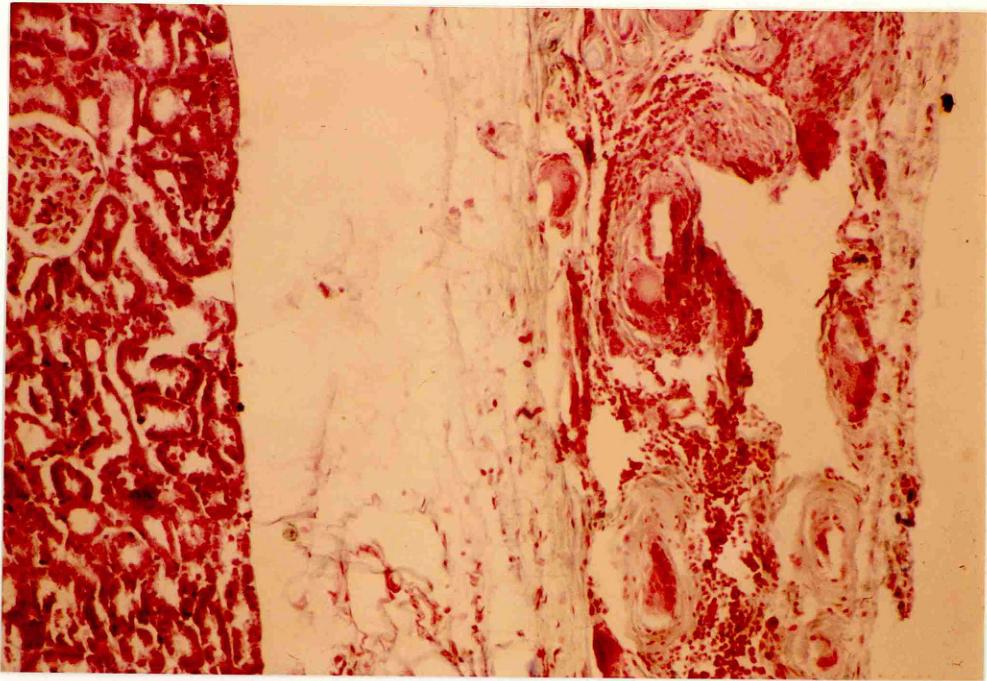
No attempt was made to induce diabetes neither were any immunosuppressive regimes used.

Results

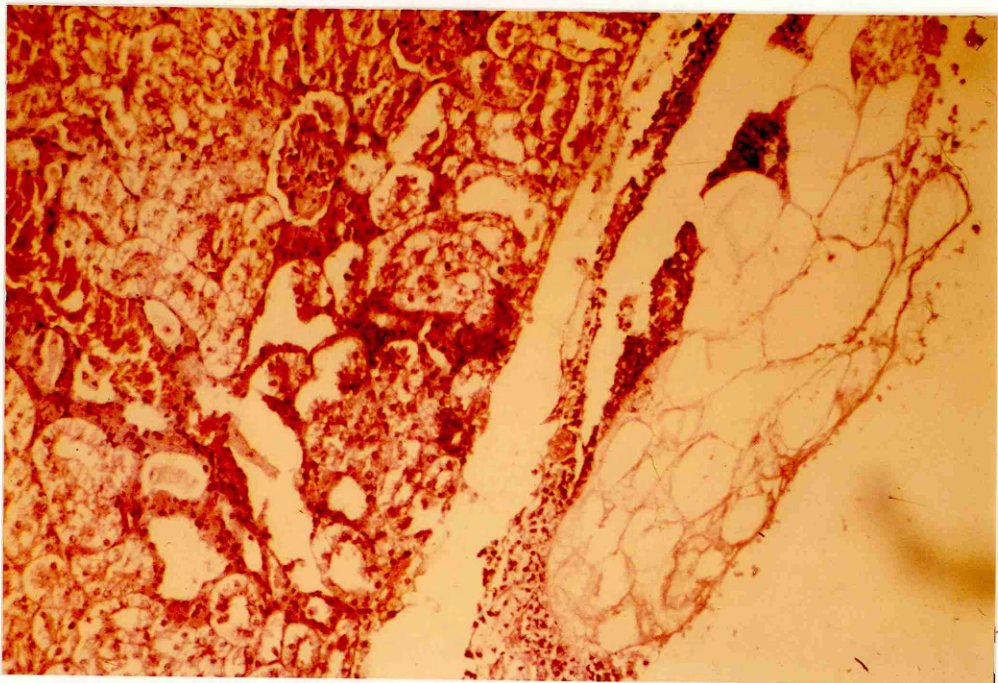
Of the 4 rats which received fresh islets two were sacrificed at 2 days and the remaining two at 4 days . The two rats which received 28 day cultured islets were sacrificed at one and three months.

In the rats which received fresh islets macroscopic evidence of graft survival was seen at day 2 but not at day 4 . Histology of the grafts revealed evidence of lymphocyte infiltration and cellular destruction at day 2 (Photograph 28) . No evidence of remaining pancreatic tissue was seen by day 4 although areas of massive lymphocyte infiltration were noted and provided suggestive evidence that the tissue had been destroyed by an immune reaction (Photograph 29).

In the 2 rats which received long term cultured islets the



Implant of Non-cultured Islets
After 2 days
Haematoxylin & Eosin Magnification X 100
PHOTOGRAPH 28

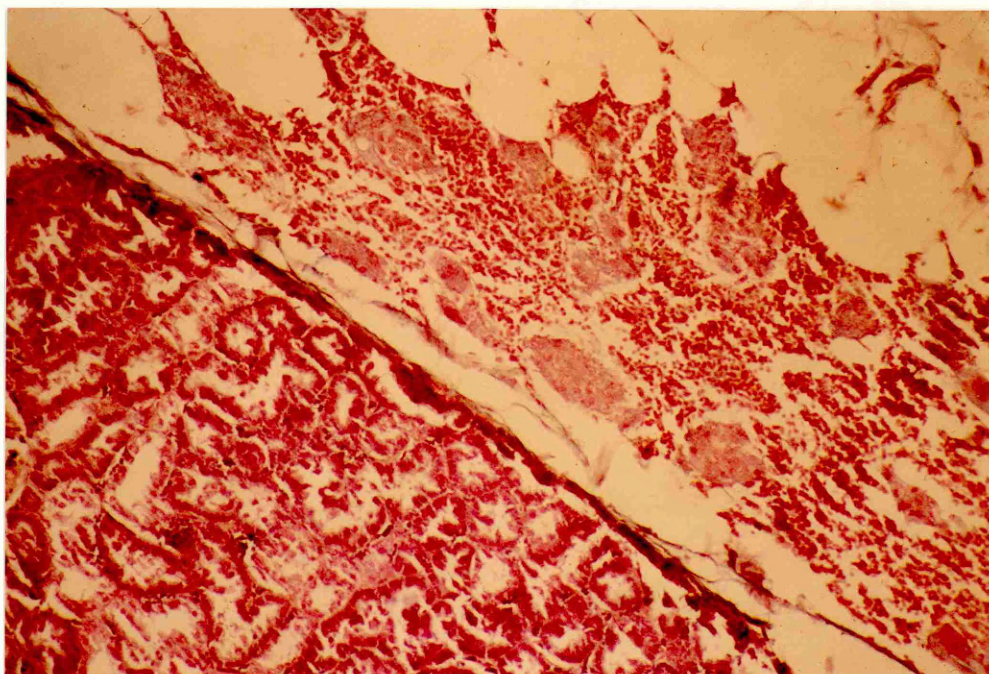


Implant of Non-cultured Islets
After 4 days
Haematoxylin & Eosin Magnification X 100
PHOTOGRAPH 29

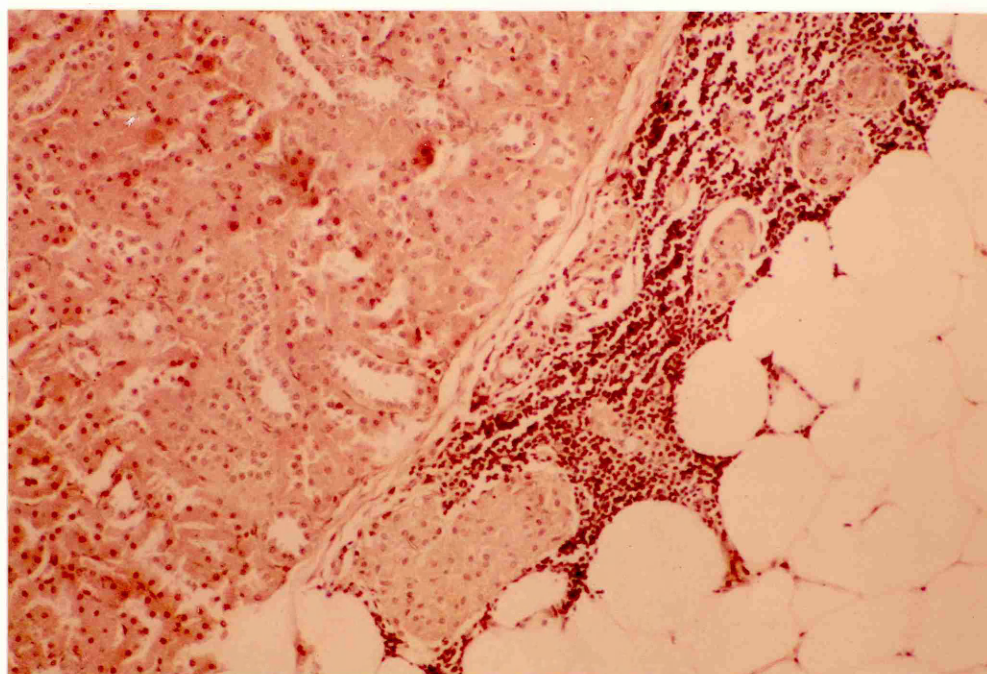
most remarkable finding was the vast amount of adipose tissue which had developed around the site of implantation. Histological examination of the tissue at the site of implantation of both the 1 & 3 month xenografts showed islet like structures surrounded by but not infiltrated by a large number of lymphocytic cells (photographs 30 & 31). Similar findings i.e. lymphocyte accumulation around surviving islets have been reported following other xenograft studies (Lacy 1982) . Staining with Ivic's Victoria Blue B (appendix II) provided proof that these structures were indeed islets and showed the presence of B cell intracellular granules within these islets (photograph 32).

One finding although not relevant to the purpose of this thesis is of interest . Following implantation of human islets all rats exhibited a significant level of hyperglycaemia ,with a mean non-fasting blood sugar of 11.9 mMol/l against a pre-operative (fasting) glucose level of 4.8 mMol/l and a non-fasting level of 5.7 mMol/ l in the two control animals. This level of hyperglycaemia would in many studies be indicative of the existence of the diabetic state and are of course in direct opposition to the aims of this project which are of course to cure and not cause diabetes . Suppressed islet function of host animals following implantation of an insulinoma has been previously reported (Like 1977) however the reasons why diabetes should occur are puzzling.

This to my knowledge is the first report of successful implantation of adult islets across a major histocompatibility barrier and although it does not prove that allogeneic human islet immunogenicity can be abrogated following culture (for



Implant of 28 Day Cultured Islets
After 30 days
Haematoxylin & Eosin Magnification X 100
PHOTOGRAPH 30



Implant of 28 Day Cultured Islets
After 90 days
Haematoxylin & Eosin Magnification X 100
PHOTOGRAPH 31

other histocompatibility barriers may exist) it does clearly demonstrate that the immunogenicity of human adult islets can be altered during culture .

These findings indicate that the culture of human purified islets may provide considerable advantage in dealing with the rejection phenomena .

CHAPTER EIGHT

SUMMARY AND DISCUSSION

The history of diabetes is fascinating and in many ways parallels the history of our knowledge of physiology .

It is clear that in antiquity the physical signs of the disease were known and described . The massive diuresis and wasting away of both muscle and fat (i.e. the exaggerated effect of starvation despite a voracious appetite)were well known .

It is now nearly 100 years since the association between diabetes and the pancreas was finally accepted and the essential role of insulin in maintaining glucose homeostasis was proposed . The expertise from many academic roots have combined to explain many of the physiological mechanisms involved .

Many of the cardinal symptoms of the disease i.e. hyperglycaemia , glycosuria , polyuria and weight loss may be explained by a lack of glucose transportation from the blood into the cells of insulin sensitive tissues i.e . skeletal muscle and adipose tissue . This diminution in intracellular glucose transport , if uncorrected , results in alternative metabolic energy pathways such that fatty acid formation and protein catabolism occur .The net result of this is to release end products into the circulation which explains the resultant negative nitrogen balance , keto-acidosis , leading to coma and death .

The repercussions of the lack ,or alterations in the effect ,of insulin are manifest in the development of the heterogenous disease which we know as diabetes .

other histocompatibility barriers may exist) it does clearly demonstrate that the immunogenicity of human adult islets can be altered during culture .

These findings indicate that the culture of human purified islets may provide considerable advantage in dealing with the rejection phenomena .

For the severely diabetic individual the discovery of the therapeutic use of insulin proved a major breakthrough in the treatment of the disease .

Innovations in the chemical structure and alterations in the time and mode of insulin therapy have combined to improve metabolic control . However , the quality of life for many diabetics , particularly those who develop the disease early in life , remains depressingly bleak.

More than 60% of juvenile diabetics will experience the serious secondary complications of the disease , the cause and prevention of which remain the outstanding problem in diabetic treatment and management .

It has been suggested that greater control of blood sugar levels may influence the onset and development of these secondary complications of the disease . The role of insulin treatment regimes designed to improve control of glucose metabolism remains contentious and both opponents and proponents of greater control continue to argue their respective cases . Whether the therapeutic use of insulin can ever achieve the sensitive control of glucose metabolism exhibited by normal individuals must remain questionable . Even if this is achieved we cannot be sure that other metabolic aspects of the disease (some perhaps as yet unknown) may influence the onset or severity of these dreadful sequelae to the disease.

In contrast there is now little doubt that total pancreatic endocrine replacement following transplantation is capable of correcting the physiological aberrations of the disease . In

addition one of the most exciting aspects of experimental pancreas and islet transplantation has been the demonstration that similar diabetic lesions in experimental animals have been reported to stabilise or in some cases even regress following successful implantation . Thus the potential of islet and pancreas transplantation (provided that it can be made safe) is one of enormous benefit as an alternative and more important an effective treatment for the disease .

For several reasons whole organ grafting has been the most commonly carried out in man . Surgical procedures are fairly straightforward and excellent glucose homeostasis has been achieved. Problems remain however in dealing with pancreatic exocrine drainage , fibrotic reactions within the gland following atrophy of the exocrine tissue and of course in dealing with the immune tissue rejection phenomena . Despite improvements in technique the results of whole organ transplantation have continued to be extremely disappointing (Calne 1983) .

Experimental studies in rodents have shown that transplantation of collagenase isolated islets from multiple donors into syngeneic recipients is now a reliable technique for correcting experimentally induced diabetes with excellent animal survival and glucose homeostasis .

Other approaches (at least in animal models) have been shown to be possible .

The continued growth and development of foetal derived tissue both in vitro and in vivo may provide exciting possibilities for the future .

An alternative strategy developed in the canine model is

that of preparing and implanting partially digested pancreatic microfragments .

Thus considerable innovation and invention have been used to design and improve strategies of tissue disaggregation , islet isolation and implantation . It would be erroneous however to suggest that all of the problems of experimental islet implantation have been resolved .

The disaggregation procedures used to prepare both isolated islet and partially digested microfragments preparations remain limited in the quantity of islets that can be recovered .

In the extensive review of attempts at human islet implantation it can be seen that the effectiveness of these procedures which have been developed in other species have been far less successful when dealing with the separation and implantation of islet cells or microfragments in the clinical field.

It is perhaps prudent to ask the question of why this success has not been repeated in the clinical model , and more importantly to consider approaches to improve the success of this technique in the clinical field .

It is my belief that improvements can only arise following the careful appraisal of techniques designed to specifically investigate and evaluate procedures developed in and shown to apply to the human gland. Perhaps surprisingly when considering the importance of this topic there is a dearth of studies which have dealt with islet isolation from and preparation of the more compact and fibrous human pancreas. The need to critically

investigate problems associated with islet preparation of the human pancreas was therefore obvious .

In the few studies where this has been attempted , evaluation of the recovered islet function have been based upon crude and possibly unreliable measures of assumed islet numbers or islet mass. The development of an in vitro method to assess endocrine function following disaggregation is considered essential for valid evaluation of the disaggregation techniques .

The emphasis of this study was therefore in developing techniques of assessing islet function prior to and following disaggregation . Only then would we be able to critically evaluate and perhaps improve methods of disaggregating the human pancreas .In particular it was decided to pay particular attention to steps in the disaggregation procedure where the loss of endocrine tissue has been reported to be most marked , i.e. following mechanical chopping and enzymatic digestion.

Our first concern however was to define the parameters (the normal values) against which assessment of the effectiveness of disaggregation procedures could be made . Considerable between sample variation was measured which presented problems in both data handling and analysis . However , the range of tissue insulin content and the dynamic aspects of IRI release in response to alterations in glucose content were defined . In addition measures were taken to verify the accuracy of the methodologies used to arrive at these figures.

Peri-perfusion techniques enabled us to measure the loss of endocrine function and to define the most advantageous storage conditions and the time limits within which this loss of function

occurred during the period of storage .

We have also shown that intra-venous distention of the gland resulted in the anatomical separation of the endocrine-exocrine tissue (a reported pre-requisite of rodent islet isolation), and that the use of the McIlwain tissue chopper was extremely effective in providing regular size fragments with no significant degree of cellular damage.

Whether rightly or wrongly we considered that the greatest improvement in endocrine tissue survival could be achieved by judicious use of the enzymes used to effect tissue disaggregation.

In theory there are two approaches to enzymatic disaggregation . The first is to use enzymes which only digest the extracellular tissue stroma , while the alternative is to use less specific enzymes and hope that the cell membranes are more resistant than the extracellular matrix to proteolytic attack.

The primary problem with commercial collagenase is that it is an enzyme complex . The consequence of this is that it was necessary to fractionate commercial collagenase into its individual enzyme components and to evaluate each in its effect in tissue breakup . The presence of at least 6 separate enzyme components were identified . The effect of these components on tissue morphology , tissue disaggregation and cell release clearly showed that only the enzyme exhibiting collagenolytic activity was essential in achieving tissue disaggregation and islet separation. In addition it was found that enzyme contaminants of crude collagenase could result in a marked degree

of cellular damage .

It is our belief that the use of a pure collagenase preparation together with a protease inhibitor provided considerable advantages in its ability to provide a viable islet preparation from the human pancreas . For this reason a disaggregation procedure was developed using this highly purified enzyme preparation .

The only real test however of a disaggregation procedure is in the total insulin producing capacity of the recovered tissue .

Peri-perfusions were carried out and demonstrated that basal IRI release during the period of peri-perfusion approximated to a mean of 1.5 Units per hour per pancreas . This was on average increased to 4.5 Units per hour under 16.8 m Mol / l glucose stimulation . This figure indicated that in excess of 60 % of the endocrine function of the pancreas could be recovered.

It was therefore considered that the endocrine components contained within the unseparated disaggregate should be sufficient to significantly alter the diabetic state following implantation.

The disaggregation technique developed using the human pancreas was shown to be equally effective when dealing with the canine gland. It was argued that its possible effect upon altering the diabetic state or possible noxious effects of implanting the unseparated disaggregate in dogs , would be predictive of its possible future application in man.

In a series of nine experiments in dogs the non-separated disaggregate was implanted as autografts into either the liver or spleen .

In each of these dogs early there were extreme fluctuations in blood glucose levels following implantation and blood sugar levels were difficult to control . In none of these animals was there a sustained beneficial effect upon blood sugar control .

Intra-hepatic implantation resulted in the development of a fatal portal hypertension . Following implantation into the splenic vasculature the resultant morphological evidence suggested that severe hepatic trauma had occurred . In addition in all dogs severe hamatological disorders resembling DIC occurred and 3 dogs died as a result of massive reactive haemorrhage .

It is at first difficult to reconcile this finding with other studies . Many investigators have used this technique in both experimental animals and man without reporting the development of serious complications , although others have been less successful . We can only suggest that two factors may have played an important role . In this study the large volume of disaggregated tissue (15 to 30 g) which was implanted may have produced a more pronounced effect upon the liver and blood homeostasis . In addition it is known that enzyme contaminants of collagenase have been shown to induce cytotoxic changes within exocrine cells and it may be that the exocrine components of the implanted tissue were more viable and exerted a greater degree of pancreatic exocrine release resulting in increased cell damage and activation of the blood coagulation cascade . Whatever the validity of this this the extremely disappointing results indicate that the risks involved in implanting this type of

unseparated digest were considerable .

As mentioned previously the rationale behind pancreatic transplantation suggests that its greatest potential may be in the prevention of the sequelae of secondary complication of the disease . In order to meet this aim it must be shown to be safe and effective .

It was clear that steps were needed to eliminate the toxicity of this type of pancreatic disaggregate before this aim can be met.

Techniques of harvesting isolated islets developed in animal models were shown to be inadequate in dealing with the human pancreas . An alternative techniques which exploits differences in the chemotaxis of islet tissue was shown to be extremely effective in arriving at a pure endocrine preparation .

Recovery of islet tissue showed that 75 % of the endocrine tissue was lost during the disaggregation and isolation procedure and it remains unknown whether this volume of endocrine tissue would be sufficient to fully restore normal carbohydrate control or whether improvements in harvesting or the possible use of islet tissue from multiple donors are needed before any significant effect on carbohydrate control can be predicted .

The long term culture studies have shown that culture as a means of human islet preservation is a possibility and perhaps more importantly has allowed us to carry out preliminary studies into whether long term culture of islet tissue may sufficiently alter the immunogenicity of islet tissue so that its possible future application in non- immunosuppressed diabetics may ethically be considered . The results of these xenogeneic studies

are encouraging in that they clearly demonstrate that the Summerlin hypothesis may also apply to the transplantation of human endocrine tissue .

In conclusion , the experimental aims of this thesis have in part been achieved . A satisfactory method of human pancreatic disaggregation has been described allowing recovery of in excess of two thirds of the endocrine function. A method of harvesting a pure endocrine tissue preparation has been shown ,however time has not as yet allowed its evaluation following implantation . Evidence from in vitro culture suggests continued function and survival of the endocrine tissue . Although studies into the optimal conditions of in vitro culture have only just begun evidence suggests that long term vitro culture may also abrogate the immune tissue rejection phenomena of human tissue sufficiently for implantation to eventually be possible in non-immunosuppressed recipients.

The implications of these studies for the possible eventual treatment of diabetes by islet implantation are clear. As mentioned previously its greatest potential must be in the treatment of young diabetics in whom the secondary complications of the disease are only becoming manifest and in whom a reasonable chance exists that these sequelae may be avoided .

The risks of implantation together with the risks involved in measures taken to avoid the immune tissue rejection phenomena must be carefully weighed against the projected life expectancy using alternative forms of treatment .

It is clear that progress in this field is being made and

that a safe and effective cure for the disease will one day be possible . In the words of Victor Hugo it is my belief that ,

' nothing is as powerful as an idea whose time has come '.

The work contained in this thesis and by other workers in this field show that although improvements are still necessary to improve the yield and effectiveness of islet implantation the promise of this technique as a complete cure for this distressing disease is as great as ever .

ACKNOWLEDGEMENTS

There is no doubt that this study could not have been completed without the generous help and advice of Dr. Richard Holmes .

I owe a special debt to the regional transplant surgeon Mr. Michael Bewick who read every word of this script , criticized firmly but fairly and greatly improved the final product . I am also deeply grateful to Mr. Bewick for supplying the human material on which this study is based and for also carrying out the canine surgery with great skill and dexterity .

I am grateful too to Mr. Chris Rudge who also provided human pancreatic tissue and to Mr. Lawrence Armitage who kindly and expertly prepared the histology .

I would like to also thank the Tissue Typing Dept. of Guy's Hospital who supplied many of the materials used.

Finally I would like to gratefully acknowledge the help and understanding of my wife without which this study would not have been possible .

APPENDIX I

MATERIALS

Source of Tissue ,

Rats and dogs were obtained through and experiments carried out at the Royal College of Surgeons Research Centre or the animal house at Guys Hospital.

Adult and infant human pancreatic tissue was obtained following nephrectomy of brain dead kidney donors. The donor age ranged from 3 to 70 years. In all cases the pancreas was stored in ice until processing could be carried out. The period of warm ischaemic time was limited to less than 5 minutes. There was however a considerable and largely unavoidable variation in the period of cold ischaemia due to the time taken to transport donor tissue etc. This ranged from 30 minutes to in excess of 8 hours.

Apparatus

An orbital shaker (type TM-1) was purchased from Braun .The McIlwain tissue chopper was supplied by Mickle Laboratories , Gomshall , Surrey . The modular incubator and multidish culture dishes were obtained from Flow Laboratories. All other apparatus was standard laboratory equipment.

Chemicals

Insoluble collagen type I , azocasein , collagenase type IA and 5 , N -Benzoyl-dl-arginine p-nitroanilide ,bovine albumen fraction V (RIA grade) , α -1-antitrypsin, PGO enzymes , o-dianisidine , 3-isobutyl-1-methyl-xanthine and dl-dithiothreitol were obtained from Sigma . Ultragel AcA 54,44,34 and 202 and ampholines pH 3.5-10 were supplied by LKB and Sephadex 200

superfine, Percoll and Ficoll were purchased from Pharmacia. Flow Laboratories supplied collagenase type IV , foetal bovine serum and RPMI 1640 culture media . Gibco-Biocult prepared RPMI 1640 without glucose , arginine, leucine , glutamine and cysteine as a special order. Antibiotic-antimycotic soln. and L-leucine, L-arginine, L-cysteine and L-glutamine were also supplied by Gibco-biocult. Anti-insulin serum and insulin binding reagent were purchased from Wellcome Reagents , and glucagon antisera was kindly donated by Surrey University. Human insulin and glucagon was given by the National Institute for Biological Standards and Control . Iodinated insulin was purchased from Amersham International and iodinated glucagon from New England Nuclear. Diazoxide was supplied by Allen and Hanbury's .

APPENDIX II

Methods

II. a LABORATORY TESTS

Insulin

Insulin or more accurately immunologically reactive insulin activity (IRI) was assayed according to the double antibody method developed by Hales (1963) . The assay was carried out using the pre-precipitated double antibody (binding) reagent and following the method recommended by Wellcome Reagents Ltd. .All samples standards and blanks were assayed in duplicate .IRI activity was estimated from the mean of two standard curves (at the beginning and end of the assay)prepared using the international standard for human insulin supplied by the World health Organization .

The diluent used to prepare standards and tissue extracts is given in Appendix III . Non-specific binding effects ($200\mu\text{l}$ of diluent plus $100\mu\text{l}$ label) were less than 1% in all assays .It is possible that non-specific factors (i.e.plasma effects) could be present when assaying IRI in plasma samples but the pre-precipitated double antibody method was chosen in part for its known insensitivity to these artifacts.

Figure A1 shows a typical standard curve . Per cent counts precipitated are shown on the ordinate and Log microunits of insulin standard are on the abscissa.

The IRI content of the unknowns (mean percentage bound of the two duplicates) were read directly from the steepest and therefore most sensitive part of the standard curve (between 2 and $128\mu\text{U/ml}$).

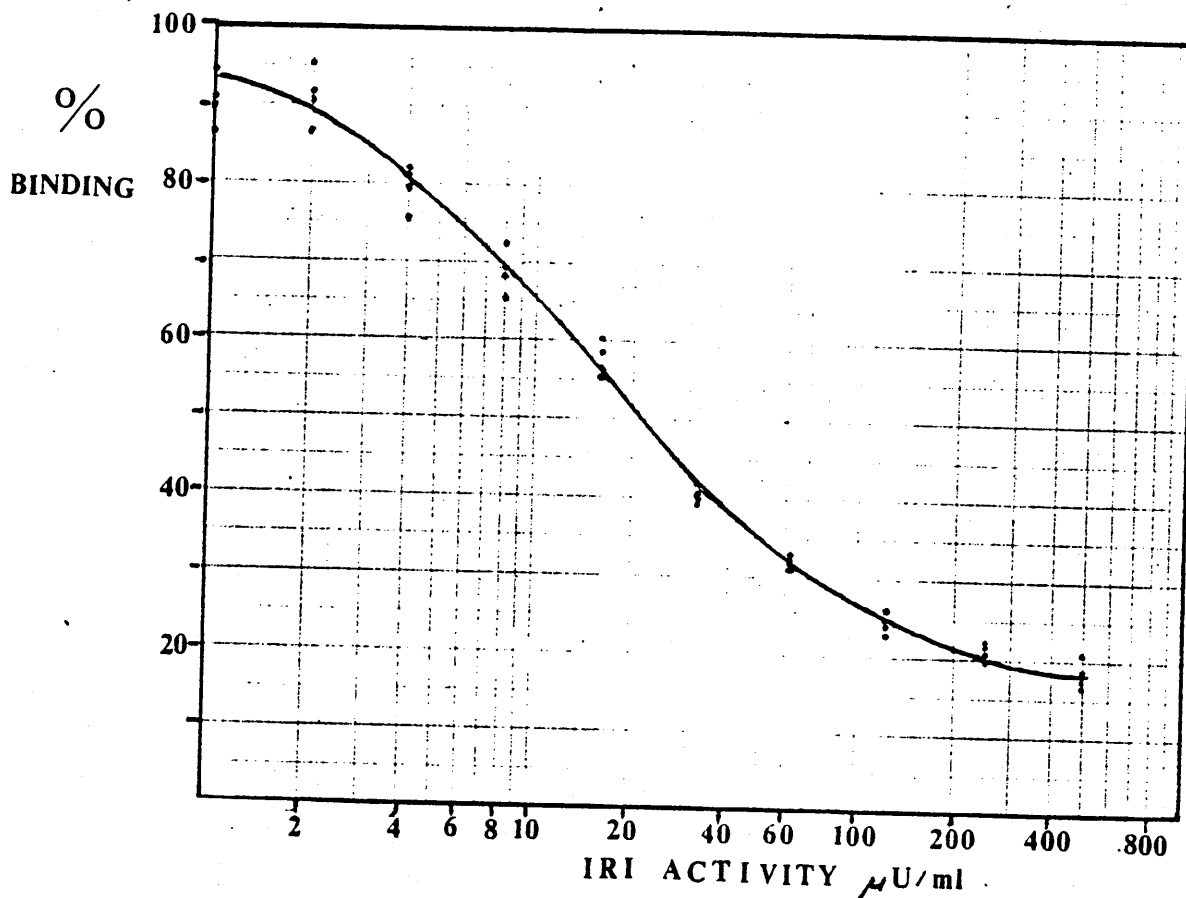


FIGURE A 1

The reliability of estimation was initially confirmed by the quality control scheme organized through the Dept. of Biochemistry at the University of Surrey. In addition aliquots of three pooled and frozen serum samples with known IRI concentrations were included in each RIA assay to enable between assay variation to be quantified.

The standard error of the means of the percentage binding of individual values (Figure 1) range from 0.22 to 1.8 over the range 2 to 128 U ml which indicates that the precision of within test accuracy of IRI provides a 95% confidence level of between $\pm 0.83\%$ at high IRI and $\pm 3.6\%$ at low IRI concentrations. Similarly the standard error of between test variation (as determined from quality control samples) was $\pm 4.2\%$ (n=12).

Because of the convention used in the literature of using metric quantitation for insulin content and international units

to report physiological levels , both have been used . These figures may be converted using the following formula.

1 Unit (4th International Standard) = 41.67 g

Total Protein

This was measured using Peterson's (1977) modification of the Lowry reaction .

Amylase

Amylase was assayed according to the method of Gomieson (1969). The assay was carried out at 25 C using hog pancreatic amylase as the standard .

Glucagon

This was measured using the radioimmunoassay method developed and supplied by Al Tagar at the radioimmunoassay department of the University of Surrey .

Plasma Glucose

Glucose was assayed using the glucose oxidase - peroxidase reaction and o-dianisidine as the oxygen receptor using kits supplied and following the procedure recommended by Sigma .

Potassium

Plasma potassium levels were measured using an Eel flame photometer calibrated using a 5 m Mol KCl / 130 m Mol Na Cl standard solution.

II.b Haematological Tests

Blood samples of 2.5 ml were collected into tubes containing 0.25 ml of 3% sodium citrate , mixed gently and stored in ice. The tubes were centrifuged at 2,000 g for 10 minutes and the plasma withdrawn using a plastic pipette. All tests were performed in

duplicate.

Prothrombin time (PT)

This was measured according to the method of Quick (1935).

Kaolin-Cephalin time (KCT)

100 μ l of plasma was mixed with 100 μ l of a 5mg/ml kaolin solution pH 7 . This was incubated for exactly 9 minutes 45 seconds at which point 100 μ l of 10 mg/ml platelet extract was added . At exactly 10 minutes 100 μ l of 0.025 Molar calcium chloride was added and the time taken for clot formation was timed using a stopwatch.

Platelet counts

These were estimated using sequestered whole blood and were counted using a model S-Plus Coulter counter (Coulter Electronics Ltd.) .

II.c Histological Procedures

The tissue was fixed in buffered formal saline (pH 7), followed by routine processing using paraffin wax. Sections were stained using haematoxylin and eosin, aldehyde fuchsin (Gommori 1940) or victoria blue 4R (Ivic 1959) .

II.d Tissue components

Insulin

Acid ethanol extraction of tissue insulin was carried out according to the method of Dickson (1977) with the following modification. Because degradation of insulin was found to be rapid in aqueous extractions the tissue was processed throughout in 80% ethanol titrated to pH3 with orthophosphoric acid. The method used is presented below .

1. The tissue was weighed and then collected into at least 4 times its volume of acid ethanol (80% Ethanol titrated to pH 3 with orthophosphoric acid and stored at 4° C) .

2. The tissue was homogenized at 4°C using an Ultra-Turax tissue homogenizer after which 0.1 Molar orthophosphoric was used to reajust to pH3 .

3. The homogenate was shaken overnight at 4° C , centrifuged at 2,000 g for 20 minutes and the supernatant saved .

4. The deposit was re-extracted using a similar quantity of fresh acid ethanol at 4 C for 6 hours . Following centrifugation the two supernatants were pooled . Two initial 1 in 100 dilutions were made using insulin diluent (Appendix III) followed by serial 1:1 dilutions . This enabled the mean of two measurements on the most sensitive part of the IRI curve to be computed..

The tissue IRI content (ng / gm / tissue) was calculated using the following formula ;

$$\text{IRI} : \frac{(\mu\text{U/ ml}) \times \text{Volume of supernatant (mls)}}{25 \times \text{Weight of Tissue}}$$

Tissue protein and amylase

Total protein and amylase was measured on aqueous tissue extracts following homogenization at 4° C using an Ultra-Turax and sonification using a MSE sonicator.

II.e Rat Tail Collagen

Acid soluble rat tail collagen was extracted following the procedures recommended by Paul (1972) . Briefly the tendons from three rat tails were carefully dissected and submerged in 150 ml of 1% acetic acid for 48 hours . Prolonged centrifugation

(1 hour at 2,500 g) was used to separate the residue from the acid solution of collagen . Before use it was dialyzed several times against distilled water .Care was taken to ensure sterility throughout .

The filters were prepared by coating with the extracted collagen following dialysis at 4°C for 36 hours and denatured by exposure to ammonia vapour . The filters were then washed in 3 changes of sterile buffered salt solution before use.

Cryopreservation

This was carried out following the procedure of Kratzsch (1982) . An equal volume of RPMI 1640 containing 20% dimethyl sulfoxide was added . The vials were closed and cooled at -2°C per minute and stored in liquid nitrogen at -196°C .

The cryopreserved tissue was rapidly thawed to room temperature using a shaking water bath .

APPENDIX III

Solutions Used

III.a Preparation of media

The choice of media to be used during storage and subsequent procedures was considered important. It was decided to use a defined culture media of which RPMI 1640 was the one of choice mainly because histological studies on pancreatic tissue incubated in either various balanced salt solutions or culture media clearly demonstrated the superiority of this defined media in terms of supporting tissue survival.

Two considerations however prompted us to prepare two different solutions by modifying the basic recipe of RPMI 1640 to suit the purpose for which this culture media was intended.

Soln 1 , Periperfusion media

Differences have been reported (Lacy 1981) in the pattern of insulin release obtained using different perfusates . This is to some extent due to the presence of insulin secretagogues when more complex media are used. For this reason the level of the two amino acids known to increase insulin secretion i.e. leucine and arginine , were reduced to near physiological levels (10 and 15 m Mol / L respectively. Bovine serum albumin (RIA grade) was added to a final concentration of 500 g / ml and the glucose concentration was adjusted to either 2.8 or 16.8 m Mol/L . Antibiotic-antimycotic solution (Gibco-Biocult) was added at the recommended level (1ml / dl) to prevent microbial contamination . All other ingredients were as recommended by Moore. (1967).

Soln.2 , Digestion solution.

The active conformation of bacterial collagenase depends upon both the availability of calcium ions and the presence of zinc within the molecule (Nordwig 1971). Collagenolytic activity has been shown to be reversibly inhibited by chelating agents such as EDTA (Gallop 1957) and irreversibly inhibited , (presumably by sequestration of the zinc atom) by amino acids containing SH groups of which cysteine and cystine have been shown to be the most effective (Harper 1970) .

For this reason cystine was omitted from the digestion solution. Leucine (10 mg/L) and arginine (15 mg/L) were added. Glucose was added to a final concentration of 2.8 m Mol/L together with 1ml/dl antibiotic-antimycotic solution .All other ingredients were as described by Moore (1967).

III.b Insulin Diluent

This was made up from the following formula using double distilled water ;

1. 4.6 g $\text{Na}_2 \text{HPO}_4$
2. 1.9 g $\text{NaH}_2 \text{PO}_4 \cdot 2\text{H}_2\text{O}$
3. 9.0 g NaCl
4. 1.0 g Sodium Azide
5. 5.0 g Bovine Serum Albumin (R.I.A. Grade)

PERIPERFUSION SYSTEM

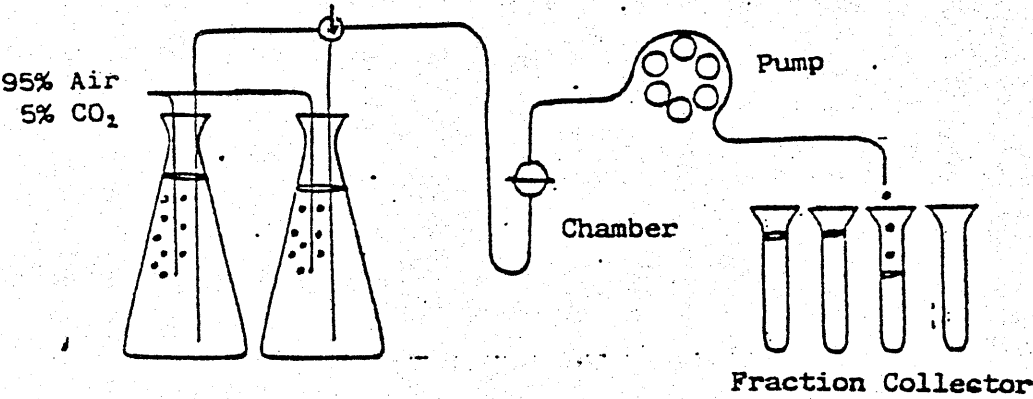


FIGURE A.2

APPENDIX IV

IV.a Periperfusions .

Basically the periperfusion system consisted of a 20 m nylon mesh filter (R.Cadish , London) enclosed within a polycarbonate filter holder (type 165 , Sartorius) through which the periperfusion solution (page 175) was drawn at a constant rate using a multichannel Serva elution pump , (Figure A.2) .

Care was taken to avoid the introduction of air into the perfusion system as this was noted to result in surges in insulin release. For this reason a 3 way tap was used so that chopped tissue or tissue disaggregate could be introduced , and the perfusate changed without introducing air bubbles. Glucose was added to a final concentration of 2.8 or 16.8 m Mol. The perfusate was constantly gassed with a 95% air 5% CO gas mixture and the perfusion was carried out within a 37°C incubator , thereby ensuring adequate temperature control.

The perfusate was collected at room temperature using a LKB Ultrarac fraction collector and stored at -20 ° C until it could be assayed for insulin.

APPENDIX V

Enzyme Preparation

Methods used

Although many separation techniques have been employed in isolating the various enzymes from the filtrate of *Clostridium histolyticum* (Yoshida 1965 , Kono 1968 , Kesselring 1971) it was decided to apply gel filtration both for its ease of application and because the reported recovery of enzyme activity has been extremely high (Mandl 1961).

Gel Filtration .

Collagenase from various suppliers was chromatographed at room temperature on either a 2.5 X 100 cm column using sephadex G200 or on three 2.5 X 70 cm columns connected in series . These columns contained Ultragel Aca34 , Aca44 , and Aca 44 . The elution buffer used was 0.75 M tris HCl, pH 7.3 , containing 0.4 mg / ml sodium azide to prevent microbial contamination. The flow rate was 40 ml per hour . Ultra violet absorption was measured at 278 nm .

The elution buffer and sodium azide was removed prior to use by eluting fractions through Ultragel Aca 202 equilibrated with the media to be used to digest the tissue .

Electrofocusing .

This was performed using a LKB 8100-1 preparative column and a glycerol gradient containing ampholines covering a pH range of between 3.5 and 10.

Enzyme analysis ,

Collagenolytic enzymes have been defined as those enzymes which specifically degrade soluble and non-soluble collagen and model peptides of the (Gly - Pro - r) type , under physiological conditions.

This definition is complicated in that enzymes have been described which attack native and denatured collagen but which are unable to cleave model peptides (Straub 1966) . Similarly (Mitchell 1968) has described a 'pseudocollagenase' from *Clostridia histolyticum* which cleaves synthetic gly-pro-n peptides but is inactive against native collagen. In addition it is known that a heterogeneity exists among collagenolytic enzymes derived from *Clostridia histolyticum* in terms of the number of collagen peptide bonds hydrolyzed and in their enzyme kinetics against differing substrates (Nordwig 1971).

The choice of substrate for measuring collagenase activity is therefore very important and to some extent is dictated by the use for which the purified enzyme is intended . It was considered that in attempting to effect tissue disaggregation the enzyme presumably acts upon native collagen and for this reason bovine insoluble collagen was chosen as the substrate .

Collagenase activity was measured using the method of MacLennan (1953) at 37°C , except that the reaction time was reduced to three hours . The extent of native collagen breakdown was estimated by measuring free amino acids liberated according to the colorimetric assay described by Rosan (1957) and quantified against a standard curve prepared from L-leucine.

The method used is presented below.

OPTICAL DENSITY OF L-LEUCINE STANDARD CURVE

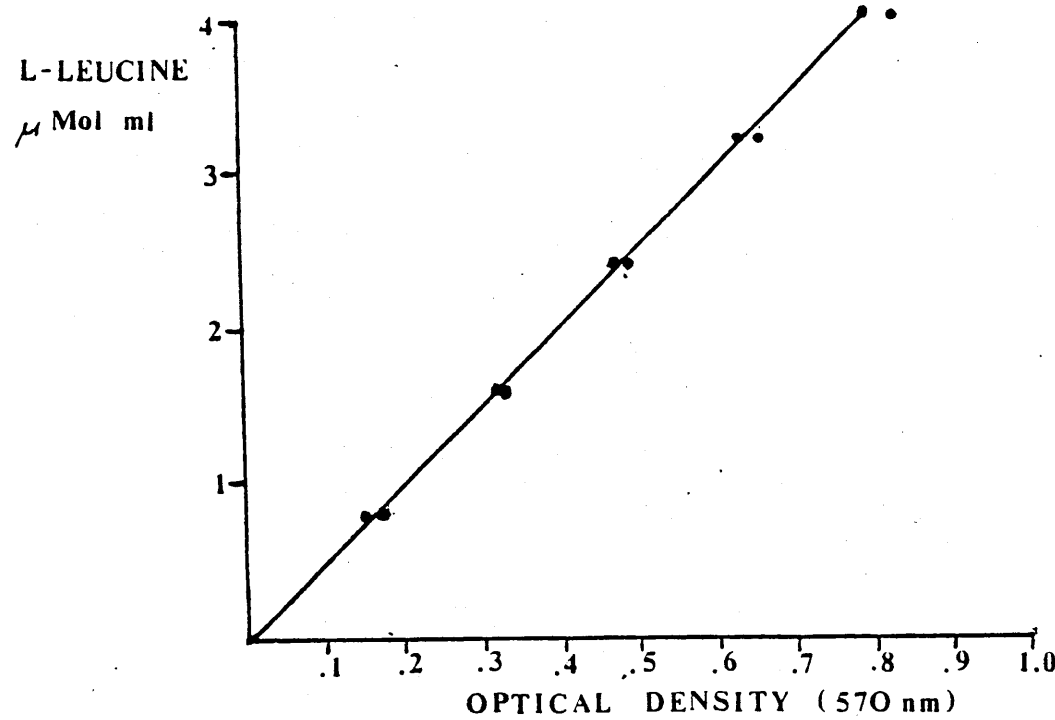


FIGURE A3

TIME COURSE OF PRODUCT FORMATION
BY COLLAGENOLYTIC ENZYME PREPARATIONS

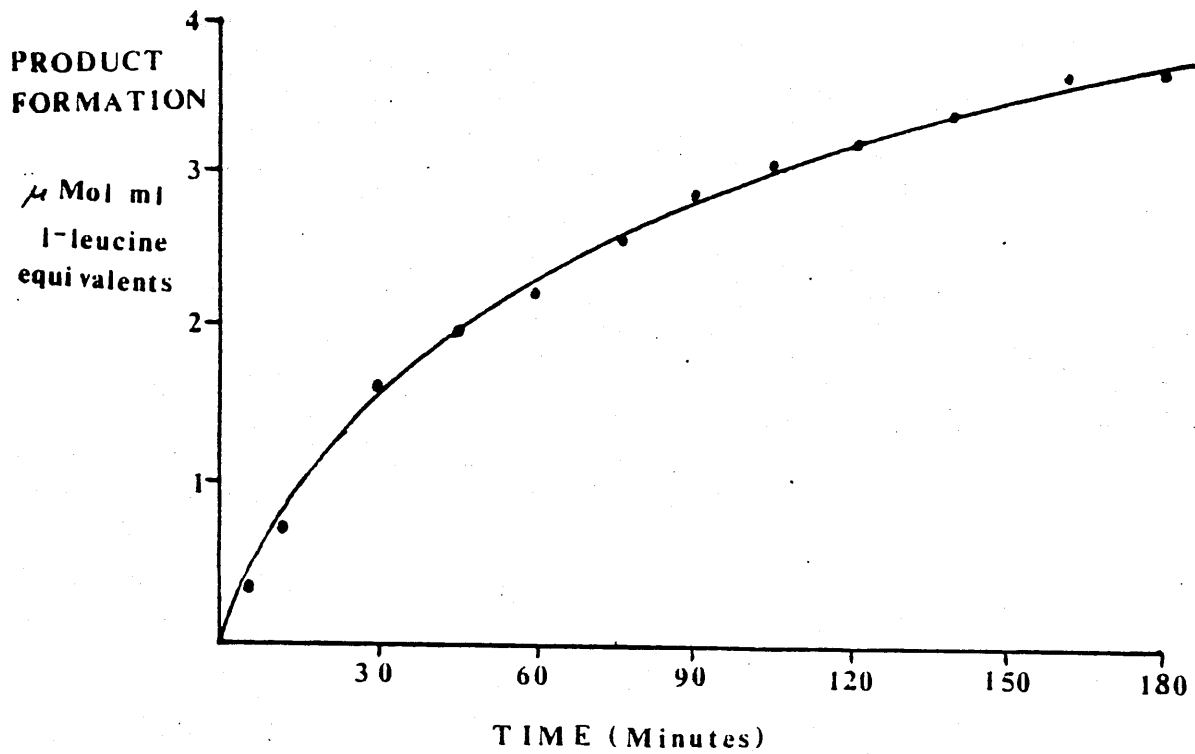


FIGURE A4

Reagents:

1. Substrate; Insoluble bovine tendon collagen (Type I , Sigma)
2. Substrate Buffer; 0.05 M Tris-HCl with 0.36 M calcium chloride
pH 7.6.
3. Inactivator; 0.1 M Ethylenediamine Tetraacetic Acid
4. Chromagen; 3% Ninhydrin in 2-Ethoxyethanol
5. Chromagen Buffer; 3.5 M Acetate-cyanide buffer , pH 5.2
(360g CH₃COONa.3H₂O , 67ml CH₃COOH to which is added NaCN to
a final concentration of 0.0002M immediately before use) .
6. Diluent; n-Propanol diluted 1:1 with water.

Method,

Weigh 25 mg substrate into test tubes for each unknown plus two tubes for blanks (no enzyme). Add five mls of soln.2 and incubate in a shaking water bath for 15 mins at 37°C. Start the reaction by adding 100 μ l of enzyme solution . After 3 hours stop the reaction by adding 500 μ l of soln.3 . Centrifuge at 2,500g for 20 minutes .

To 250 μ l of supernatant, blanks and a 0 to 4 M ml L-Leucine standard curve add 600 μ l of soln.5 and 400 μ l of soln 4. Boil for 20 minutes to enable full colour development , cool and add 10 ml of soln.6 . Read optical density at 570 nm.

Figure A 3 relates μ Moles of leucine per ml to optical density at several dilutions of leucine. Beer's law can be seen to hold at all of these dilutions.

The reaction rate was estimated by measuring product formation at several dilutions of enzyme with constant substrate concentration against time. These results are presented in (Figure A 4)

REACTION RATE OF DIFFERING DILUTIONS OF ENZYME

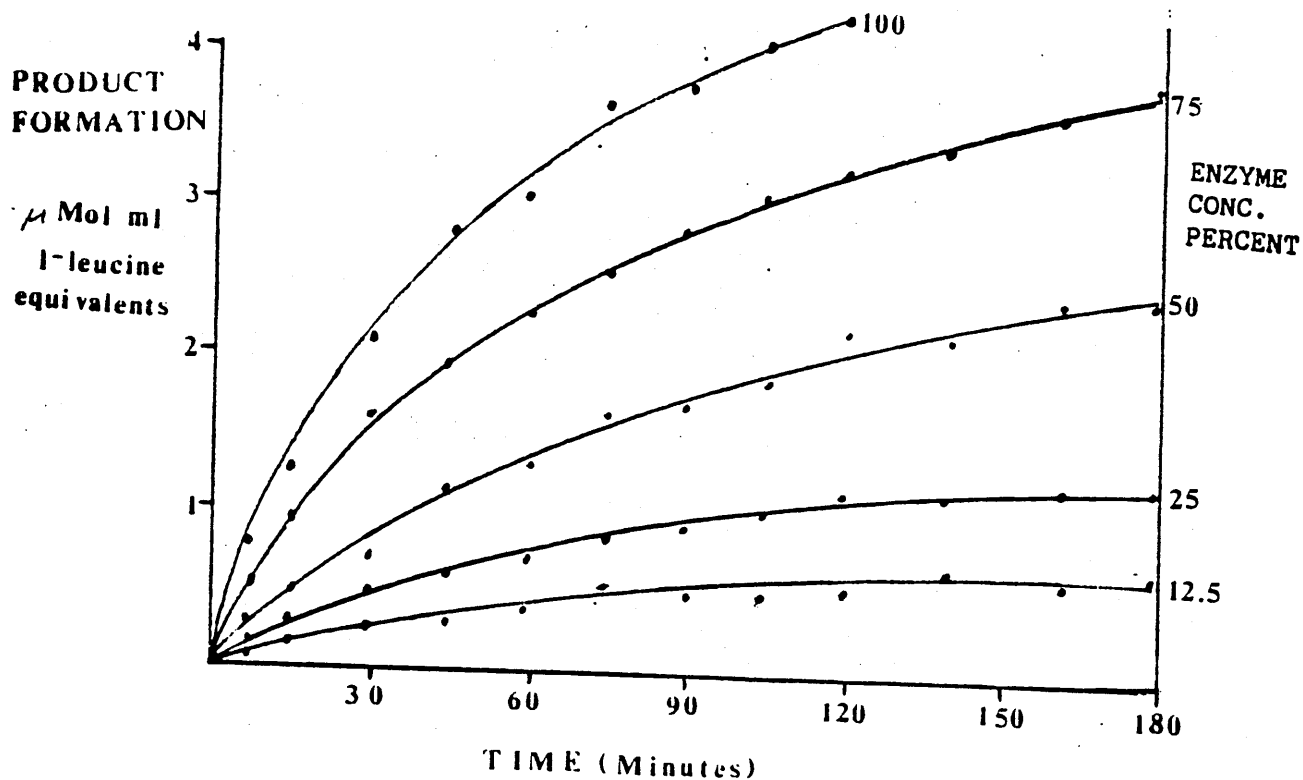


FIGURE A5

PRODUCT FORMATION AT 1, 2 and 3 HOURS USING DIFFERING DILUTIONS OF ENZYME

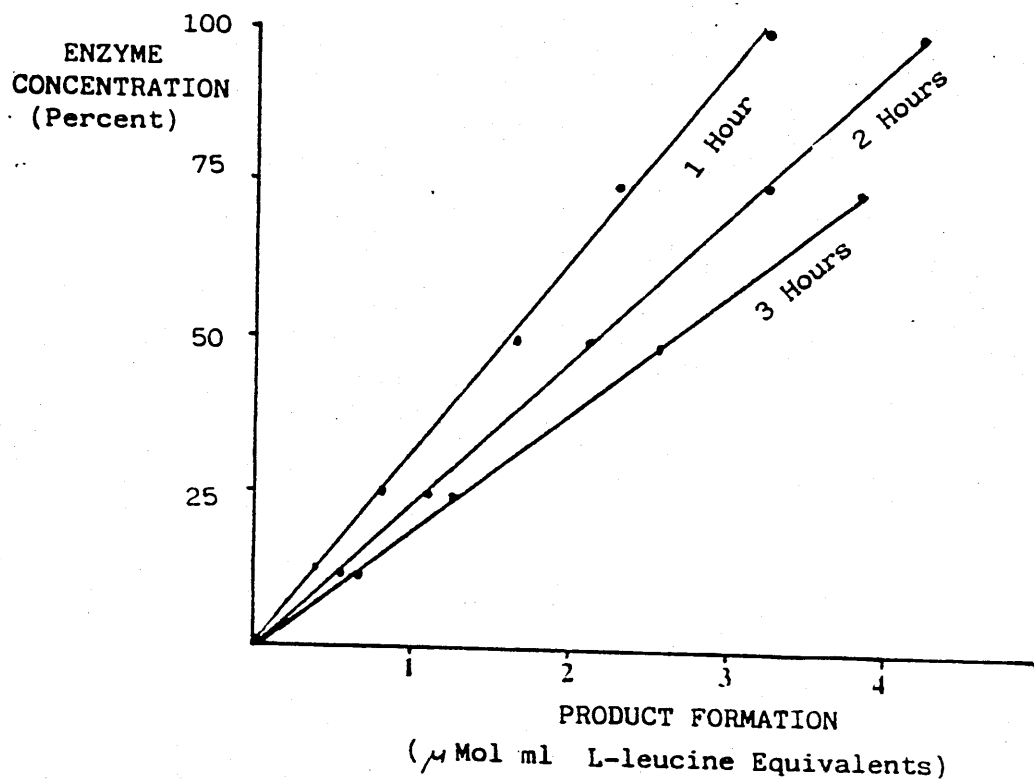


FIGURE A6

As can be seen the rate of enzyme reaction tends to be non-linear with time . Several factors may combine to produce this lack of linearity i.e. enzyme inactivation , depletion of substrate and accumulation of products etc. or it may be that the plot reflects the additive effects of more than one collagenolytic enzyme .

Figure A 5 shows the product formation at 3 hours for differing concentrations of a column fraction with high collagenolytic activity . As can be seen the shape of the curve reflecting the time course of product formation is similar at each concentration of enzyme used and more importantly , the product formation is linear with respect to enzyme concentration (Figure A 6). The amount of product formed is therefore proportional and can be used to provide a measure of enzyme activity against this specific substrate .

Results are expressed as 1 unit = 1 μ mole leucine equivalent liberated per ml enzyme solution in three hours at 37° C .

Tryptic activity was measured according to the method of Erlanger (1961) . The hydrolysis of N-Benzoyl-DL-Arginine-p-nitroanilide HCl was carried out at 25° C and at pH 7.4 . Results are expressed as μ moles hydrolyzed per ml per minute.

Caseinolytic activity (non- specific proteolytic activity) was measured using a modification of the method of Charney (1947), . The coupling of a protein with a diazotized aryl amide produces an azoprotein (in this case azocasein) . This may be precipitated with Trichloroacetic acid (TCA) to produce a colourless filtrate . If the azoprotein is subjected to

PROTEINS USED TO CALIBRATE ULTRAGEL COLUMNS

Protein Loaded	Molecular Weight	Log Mol. Weight	Elution Volume	K _{av}
Dextran	476,000	5.67	270	0
2,000,000	150,000	5.176	376	0.15
Glucose oxidase	110,000	5.041	430	0.21
Uricase	66,000	4.819	584	0.37
Bovine albumin	36,000	4.556	768	0.59
Pepsin	25,000	4.398	860	0.68
Chymo- trypsin	21,000	4.322	900	0.73
Papain	13,700	4.137	990	0.85
Ribo- nuclease				

TABLE A.1

proteolytic digestion coloured products are formed the intensity of which are a function of the activity of the enzyme solution. The assay used was as follows .

500 μ l of the enzyme solution was added to 4 ml of 5 % azocasein in 0.15 M Tris / HCl buffered to pH 7.4 and pre-warmed to 37°C. After 1 hour the reaction was stopped by adding 1 ml TCA and centrifuged at 2,500 g for 10 minutes .1 ml of the supernatant was diluted to 5 mls with 0.8 M NaOH and the Optical density was read at 470 nm . Results are expressed as 1 Unit = 1 μ mole hydrolyzed azocasien per hour at 37 C.

Total protein was assayed using the modified lowry reaction of Peterson (1977) . Bovine albumin fraction V was used as the standard.

Results.,

Column Chromatography.

The log molecular weight of globular proteins bear a linear relationship to the elution volume . The columns were calibrated using proteins of known molecular weight (Table A1) .

K_{Av} values were calculated from the following formula ,

$$K_{Av} = \frac{V_e - V_o}{V_t - V_o}$$

(where V_e = the elution volume , V_t = the total column volume and V_o = the column void volume.)

The calibration curve is shown in Figure A.7 .

Between 500 mg and 1 g of commercial collagenase from various suppliers was dissolved in 10 ml of elution buffer and introduced onto the columns .

CALIBRATION OF ULTRAGEL COLUMNS

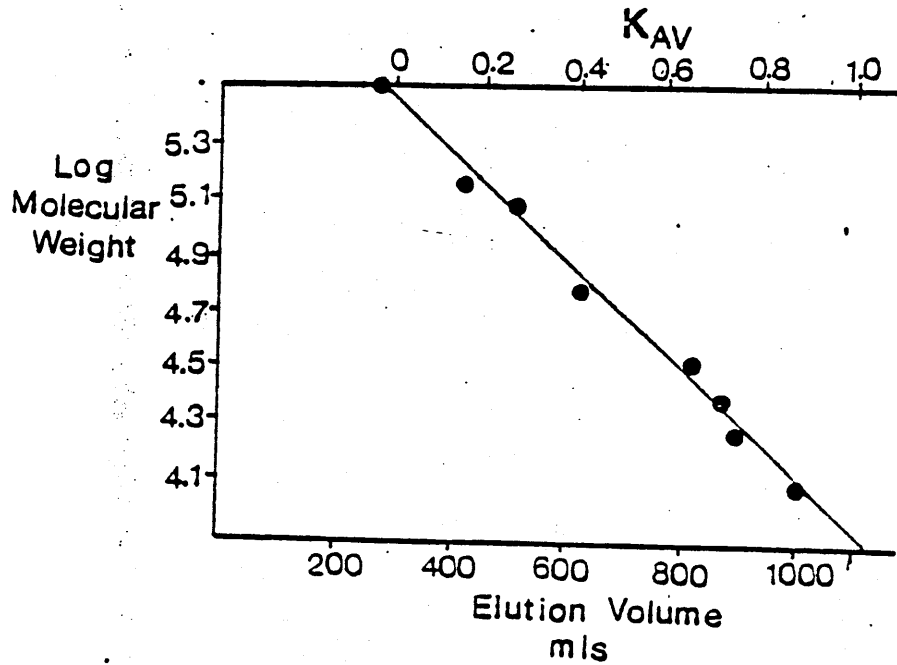


FIGURE A.7

PROTEIN CONCENTRATION

ULTRAGEL FRACTIONATION OF CRUDE COLLAGENASE

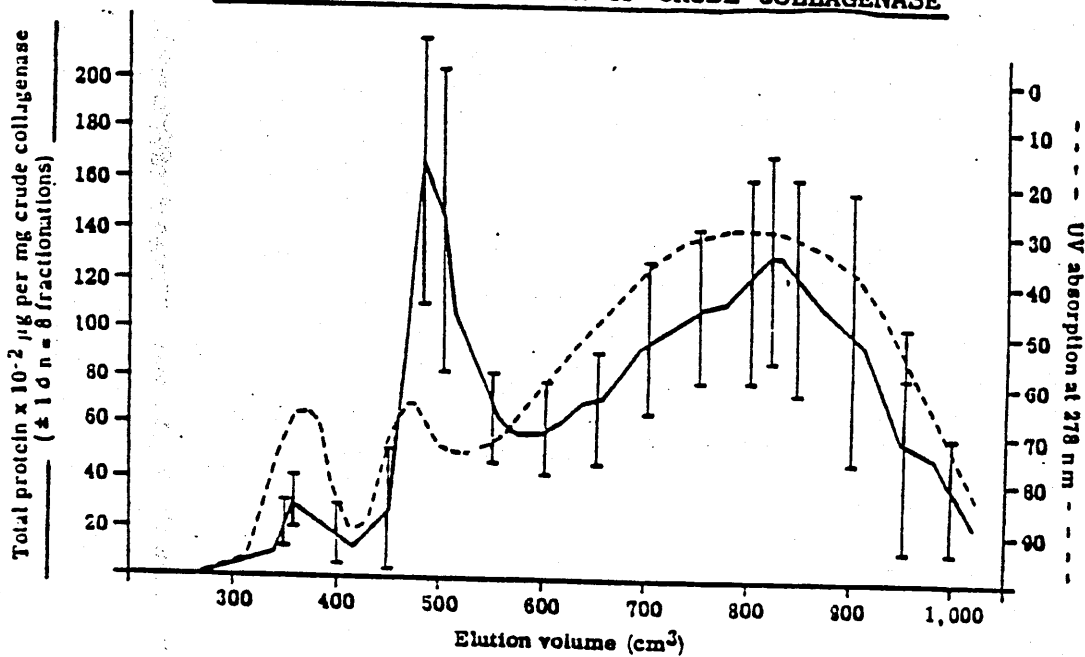


FIGURE A.8

The UV absorption spectra Figure A.8 shows two fairly narrow absorption maxima with a K_{Av} of 0.11 and 0.82 . These correspond to molecular weight proteins of approximately 170,000 and 95,000. The remainder of the eluate comprised a less well defined absorption maxima between K_{Av} 0.4 and 0.95 .

This UV absorption spectra was essentially the same with all batches of commercial preparations of collagenase tested.

From Figure A.8 it is also clear that in comparison to the UV absorption the protein spectrum showed a marked increase in those fractions at the K_{Av} value of 0.28 .

This discrepancy can perhaps be explained.

It has been shown that the UV absorption characteristics depend mainly upon peptide bonds and the presence and the degree of ionization of the three aromatic amino acid residues tyrosine, tryptophan and phenylalanine. Of these three amino acids only tyrosine and phenylalanine have been demonstrated in acid hydrolysates of collagenase and phenylalanine is known to absorb less strongly at this wavelength.

In the Lowry reaction the colour yield is considered to arise from the same three aromatic amino acid residues but also to some extent sequences of certain amino acids bearing functional side groups such as arginine, histidine and glutamic acid .

It therefore appears likely that collagenase may absorb UV light less strongly than most proteins and for this reason the Lowry reaction may provide a more accurate estimate of the protein spectra obtained following gel filtration of collagenase.

The column fractions were assayed for collagenolytic ,

ENZYME SPECTRUM FOLLOWING COLUMN
FRACTIONATION OF CRUDE COLLAGENASE

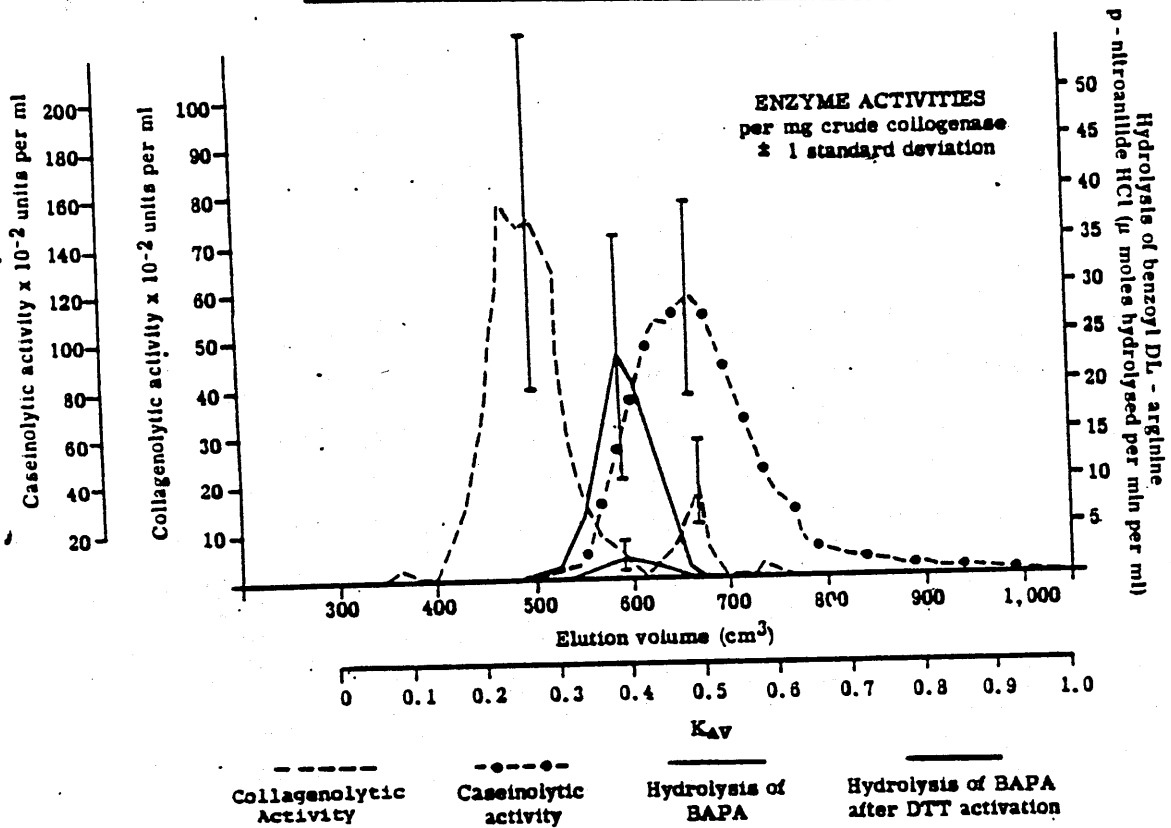


FIGURE A.9

tryptic and non-specific protease activity. These results are shown in Figure A.9 .

The first enzyme to elute was shown to coincide with the first protein peak . This enzyme exhibited only a slight non-specific activity and its characteristics i.e. high molecular weight and low K_{Av} suggest that it may be the amino-peptidase first described by Kessler (1973) .

As can be seen the main peak exhibiting collagenolytic activity appeared in the K_{Av} range 0.12 to 0.3 . This corresponds to a molecular weight of approximately 95,00 to 110,000 which is in close agreement to published figures (Yoshida 1965) . It is interesting that two distinct peaks were obtained and may correspond to the type I and type II forms of the enzyme reported by Kono (1968) . Both of these peaks eluted well before the contaminating proteases so that a pure collagenolytic enzyme preparation was obtained for use in tissue disaggregation.

The enzyme spectrum shows the presence of the tryptic like enzyme clostripain eluting between K_{Av} 0.3 and 0.5 and a broad band of non-specific protease activity eluting between K_{Av} 0.32 and 0.68 .

The tryptic and non-specific enzyme fraction were fractionated further following electrofocusing . It was found that clostripain produced a single band exhibiting a P_i of 4.8 . In contrast the non-specific protease activity could be shown to be comprised of three separate enzymes with P_i values of 3.7 , 4.8 and 7.5 (Figure A.10).

The methods presented above have enabled us to isolate

ISO-ELECTRIC FOCUSING OF NON-SPECIFIC PROTEASE

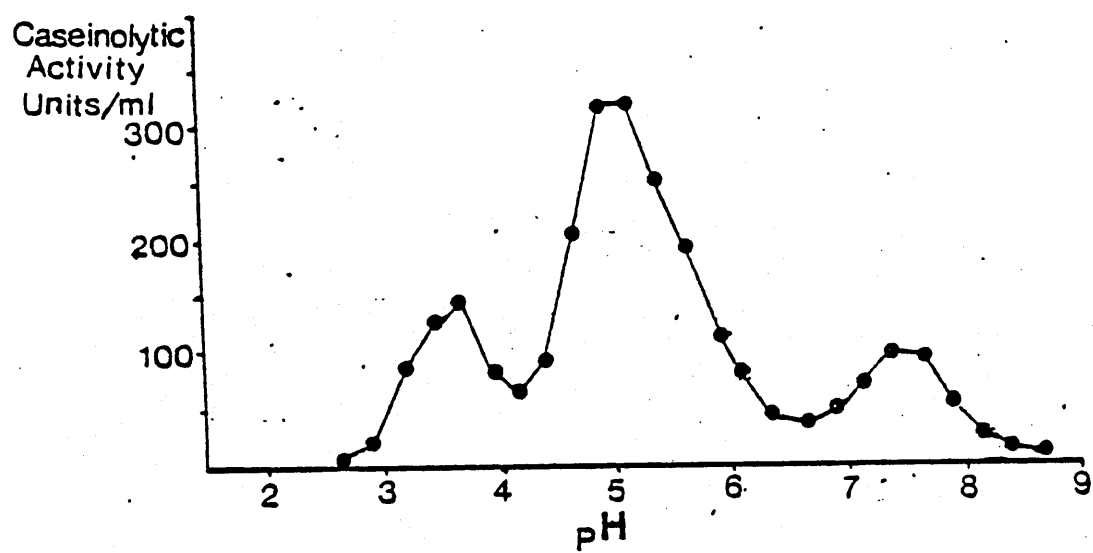


FIGURE A.10

enzymes preparations with defined activities . The purity of each of the fractions obtained following gel filtration in terms of the number of proteins or enzymes they contained would require further separation techniques (i.e. electrophoresis or affinity chromatography) or extensive experimentation into the enzyme kinetics or immunological properties contained within these fractions . These were not carried out .

The above procedures have enabled the separation of enzyme preparations classified according to their ability to react with one or each of three substrates known to be specific for collagenolytic, tryptic and non-specific enzyme activities.

This allows us to critically examine the role of these individual enzyme preparations in terms of the type of bonds they attack and their effectiveness in tissue disaggregation and in preserving cell viability .

APPENDIX VI

NORMAL CANINE VALUES

Tissue Components

TISSUE COMPONENTS OF THE NORMAL CANINE PANCREAS

	INSULIN ng g wet wt.	TOTAL PROTEIN mg g wet wt.	AMYLASE mg g wet wt.
	144,640	126	16.3
	276,970	110	27.3
	91,082	154	13.8
	74,162	161	18.9
	41,163	179	7.9
	109,966	129	11.2
	214,978	168	14.8
	91,082	142	15.9
MEAN	130,505	144	15.8
S.D	78,807	23	5.7

TABLE A.2

REGIONAL DISTRIBUTION OF PANCREATIC INSULIN CONTENT

INSULIN CONTENT OF TISSUE SAMPLES (ng per gm wet weight)			
	TAIL OF PANCREAS (Splenic Lobe)	BODY OF PANCREAS	HEAD OF PANCREAS (Uncinate Process)
	27,853	26,927	29,052
	236,152	121,335	95,380
	192,500	107,230	38,169
	178,200	57,800	65,311
	240,361	73,905	91,538
	62,430	131,389	95,363
	102,174	312,200	247,092
	346,387	166,857	67,326
	63,330	113,414	126,141
	240,361	73,905	65,311
MEAN	168,975	118,496	92,062
S.D.	102,040	79,045	61,586

TABLE A.3

PLASMA GLUCOSE LEVELS (m MOL/L) IN RESPONSE TO A 500 MG PER KG BODY WEIGHT GLUCOSE INJECTION

DOG NUMBER	MINUTES PRE AND POST GLUCOSE INJECTION													
	-10	0	10	20	30	40	50	60	75	90	120	150		
BB1	4.8	4.8	16.5	12.6	10.8	7.5	5.9	4.6	3.9	3.4	3.7	4.6		
BB2	5.3	5.6	16.0	14.0	13.1	8.5	7.8	7.6	6.5	5.3	5.6	5.3		
BB3	4.9	4.8	17.0	13.2	11.9	9.4	7.6	6.9	5.3	4.4	3.8	4.4		
BB4	4.4	4.9	14.8	12.3	8.5	8.0	6.5	6.3	5.3	4.5	4.4	4.4		
BB5	3.6	3.8	15.1	12.3	11.0	8.5	7.5	7.1	5.3	4.4	4.2	3.4		
BB6	4.8	4.8	14.7	11.5	7.4	6.6	4.6	4.4	4.4	4.4	5.0	5.7		
BB7	4.5	5.8	12.3	10.0	7.6	5.5	5.0	4.7	4.7	4.8	4.8	4.5		
BB8	5.0	5.0	12.0	10.6	8.9	8.1	6.7	6.7	5.9	5.6	5.5	5.4		
BB9	5.0	5.0	11.5	11.7	9.5	7.6	5.6	5.4	5.3	4.8	4.5	4.3		
BB10	4.5	4.5	15.2	9.3	7.1	5.5	4.8	4.9	4.8	4.8	4.9	4.7		
BB11	5.2	5.9	15.2	11.3	9.3	7.6	6.9	6.9	6.5	5.9	5.0	5.6		
BB12	5.8	6.1	12.4	10.0	6.3	4.5	4.1	5.2	5.2	5.9	5.9	5.9		
BB13	4.7	5.1	13.1	10.2	6.5	5.3	5.3	5.0	5.0	5.0	5.2	5.0		
BB14	3.9	4.7	12.3	11.0	7.2	5.9	5.0	5.3	4.7	5.2	5.2	5.8		
BB15	4.1	4.6	13.1	8.7	7.4	5.2	4.9	3.4	3.3	3.6	4.4	4.4		
BB16	4.4	4.5	10.9	10.1	6.5	5.5	4.6	4.7	4.5	4.7	5.0	4.5		
BB17	4.8	4.7	15.8	12.9	10.3	7.8	6.5	5.3	5.0	4.2	4.2	4.1		
MEAN	4.7	4.9	14.0	11.3	8.8	6.9	5.8	5.6	5.0	4.8	4.7	4.8		
S.D.	0.5	0.6	1.9	1.5	2.1	1.5	1.2	1.2	0.8	0.7	0.8	0.7		

TABLE A.4

PLASMA INSULIN LEVELS (μ U/ML) IN RESPONSE TO A 500 MG PER KG BODY WEIGHT GLUCOSE INJECTION

DOG NUMBER	MINUTES PRE AND POST GLUCOSE INJECTION														
	-10	0	10	20	30	40	50	60	75	90	120	150			
BB1	9	7	42.5	35.5	30.5	30.0	13.5	14.5	21	19	9	5			
BB2	9	9	102	86	37	34	21	9.5	9	6	7	5			
BB3	7.5	8	25.5	24.5	23.5	19	13	10.5	6.5	12.5	12.5	10			
BB4	9.5	7	126	35	37	32	33	39	20	16	11	14			
BB5	7.5	8	24	23.5	18.5	17	8.5	7.5	7	6.5	8	7			
BB6	5.5	8.5	21	20	17	15	9	5.5	5.5	12	4	9.5			
BB7	8.5	7	56	46	26	25	11.5	9.5	18.5	7.5	7	7			
BB8	6	5.5	59	41	21	14	11.5	13	7.5	8.5	6.5	11.5			
BB9	10.5	4.5	25	20.5	25	23	20.5	13	16	13.5	13	9.5			
BB10	20	21	47	38	12	21	21	16	19	17	17	19			
BB11	7.5	10	58	52	43	39	30	15.5	14	7.5	9	7.5			
BB12	6	9	49	37	28	18	12	12	4	6	4	6.5			
BB13	12	17	70	36	30	16	15	16	19	32	14.5	13			
BB14	13	6	21	19	12	13	4	10	7.5	6.5	6	14			
BB15	7.5	10	21.5	14	10	7	6	4	5	5.5	5.5	6			
BB16	11.5	11	66	32	17	18	14	12.5	17	13.5	24	41			
BB17	17	17	37	28	12.5	4	6	12.5	5	6	6	2			
MEAN	9.8	9.7	50.0	34.6	23.5	20.3	14.7	13.0	11.9	11.5	9.6	10.9			
S.D.	3.9	4.5	29.5	16.8	9.8	9.4	8.1	7.5	6.3	6.9	5.2	8.6			

TABLE A.5

PLASMA GLUCAGON LEVELS (pg / ml) IN RESPONSE TO A 500 MG PER KG BODY WEIGHT GLUCOSE INJECTION

DOG NUMBER	MINUTES PRE AND POST GLUCOSE INJECTION													
	-10	0	10	20	30	40	50	60	75	90	120	150		
BB1	182	232	149	79	156	139	120	166	105	114	146	97		
BB2	94	135	89	135	105	107	123	65	38	74	128	84		
BB3	58	75	78	98	117	115	178	67	65	28	61	51		
BB4	98	79	83	68	62	36	68	79	68	82	67	112		
BB5	122	39	30	94	51	127	59	84	92	81	124	80		
BB6	265	57	39	78	94	82	33	32	45	87	35	115		
BB7	65	98	52	30	48	88	52	75	68	118	60	42		
BB8	56	46	28	78	182	145	51	310	245	95	72	81		
BB9	162	174	166	129	142	112	106	174	151	184	152	136		
BB10	43	78	21	22	29	24	20	75	55	166	22	51		
BB11	153	150	65	38	88	113	98	112	68	66	78	99		
BB12	108	256	42	70	85	76	108	161	214	274	106	124		
BB13	74	138	35	59	34	79	25	75	127	24	19	87		
BB14	80	101	64	54	65	74	42	43	117	112	152	82		
BB15	168	156	83	70	151	142	106	77	265	106	68	164		
BB16	225	260	117	179	93	176	184	215	239	270	289	235		
BB17	64	95	64	81	93	76	54	95	45	87	89	94		
MEAN	119	127	71	80	94	101	86	112	118	116	98	102		
S.D.	64	70	42	39	44	40	49	72	77	71	65	46		

TABLE A.6

PERIPERFUSION OF ENZYMATICALLY DISPERSED CANINE
PANCREAS DURING A GLUCOSE CHALLENGE

IRI RELEASE ng g tissue				
Collection Time (mins)	Glucose Conc.mMol	Mean IRI ng	Mean Deviation ng	n
2	2.8	162.0	104.2	8
4	2.8	157.5	107.6	8
6	2.8	124.1	74.7	7
8	2.8	128.6	92.0	6
10	2.8	116.4	92.4	8
12	2.8	108.2	98.0	8
14	2.8	86.8	83.1	8
16	2.8	76.5	70.5	6
18	2.8	64.6	51.8	8
20	2.8	53.1	38.6	8
22	2.8	48.7	30.8	7
24	2.8	43.3	33.2	6
26	2.8	48.5	37.7	8
28	2.8	43.6	30.4	7
30	16.8	134.3	124.5	8
32	16.8	108.6	100.3	8
34	16.8	84.1	64.8	8
36	16.8	72.7	54.3	8
38	16.8	75.0	59.6	6
40	16.8	60.8	46.3	6
42	16.8	60.2	50.5	8
44	16.8	54.3	46.8	8
46	16.8	58.6	46.5	8
48	16.8	60.0	32.1	7
50	16.8	63.2	31.1	8
52	16.8	59.4	39.7	8
54	16.8	63.8	33.0	7
56	16.8	74.2	50.6	8
58	16.8	73.8	45.6	7
60	16.8	70.6	46.2	7
62	5.6	39.8	29.5	7
64	5.6	37.0	22.7	7
66	5.6	30.8	27.0	6
68	5.6	32.5	17.4	7
70	5.6	30.1	27.3	6

TABLE A.7

NORMAL BLOOD COAGULATION FACTORS

	Prothrombin Time (secs)	Kaolin-Cephalic Time (secs)	Platelets X 10 ml
MEAN	8.4	20.2	235
S.D.	0.9	3.5	43
n	20	20	20

TABLE A.8

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